STUDIES ON THE CELLULAR BASIS OF THE IMMUNOLOGICAL DEFECT FOLLOWING THYMECTOMY IN THE MOUSE

by

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PREFACE

In accordance with Section 8 of the Regulation and Rules regarding the degree of Doctor of Philosophy within the University of Melbourne, the author has assessed the extent of his contribution to collaborative projects to be:-

(1) 50% with Dr. T.J. Barclay in the experiment described on page 128 (PFC in the thoracic duct lymph).

(2) 50% with Dr. N.S. Weiss in the experiment described on page 139 (PFC in the spleens of neonatally-thymectomized mice at various ages).

(3) 70% with Dr. J.F.A.P. Miller in the experiments described on pages 141, 149, 154 and 156 (ARC in lymphoid cell populations and ARC precursors in bone marrow and thymus cell populations).

(4) 90% with Dr. J.F.A.P. Miller in the experiments described on pages 159, 161 and 176 (the reconstitutive capacity of syngeneic cells in neonatally-thymectomized and irradiated mice).

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SUMMARY

Answers have been sought for two key questions relevant to the proposed mode of action of the thymus as a "central" lymphoid organ. Does the peripheral lymphocyte population contain a large number of thymus-derived cells and do these cells respond to antigens by producing a progeny of antibody-forming cells?

The number of small lymphocytes emerging from a thoracic duct fistula in young adult neonatally-thymectomized CBA mice was approximately 2% of that in intact mice of the same age and body weight. Multiple injections of chromosomally-marked thymus cells increased the size of the lymphocyte pool and the majority of thoracic duct cells, stimulated into division, carried the chromosome marker of the thymus cell donor. Thymectomy in adult life was not followed by a significant decrease in the output of thoracic duct lymphocytes until many months after the operation. Whole body x-irradiation resulted in a dramatic reduction in the number of cells drained from a thoracic duct fistula and, in mice protected from the lethal effects of haemopoietic failure, the reestablishment of the lymphocyte population was dependent upon the presence of the thymus. The data supports an increasing bulk of indirect evidence which, when taken in toto, strongly suggests that a large proportion of recirculating lymphocytes are thymus-derived cells or their descendants.

The number of sheep erythrocyte antigen-reactive cells (ARC) in the thoracic duct lymphocyte population of neonatally-thymectomized mice was markedly reduced when compared with the number in the population from normal mice. The bone marrow did not contain ARC but was a potent source of ARC precursors. Neonatally-thymectomized mice did not lack precursor cells in the bone marrow but apparently lacked the thymus influence necessary for the differentiation of these precursors into ARC. Further studies on the thymus-dependent

development of ARC hinted at the possibility that the entity known as "an ARC" required the presence of both thymus- and bone marrow-derived cells to express itself in terms of haemolysin production.

Neonatally-thymectomized CBA mice failed to respond in normal fashion to a primary injection of sheep erythrocytes (SRBC) and the peak number of haemolysin plaque-forming cells (PFC) in the spleen was reduced by a factor of 1 $\log_{10}$. The PFC response was increased by injections of either thymus or thoracic duct lymphocytes from CBA, (CBA x C57BL)F_1 hybrid, and C57BL donor mice. In thymectomized mice reconstituted with semiallogeneic and allogeneic cellular inocula, the PFC carried the immunogenetic characteristics of the host and not those of the inoculated cells. Hence, thymus and thoracic duct cells were not reconstitutive simply by virtue of the ability to transform into PFC.

Thymus and thoracic duct cell inocula contained "reactor cells" which responded to SRBC antigens in irradiated mice by undergoing a burst of mitosis. Thoracic duct lymphocytes, unlike thymus and bone marrow cells, were able to produce PFC when injected together with SRBC into irradiated mice. However, the PFC response in irradiated recipients of thoracic duct lymphocytes was increased substantially by a simultaneous injection of bone marrow cells. Combinations of cells from semiallogeneic mice did not interact upon transfer to irradiated recipients but clear evidence of synergism in PFC production was apparent in adult-thymectomized irradiated mice protected with CBA bone marrow cells and injected two weeks later with (CBA x C57BL)F_1 thoracic duct cells. In this case, the vast majority of PFC were derived from the bone marrow inoculum.

The results suggest that the bone marrow contains only PFC precursors, the thymus only "reactor cells", but that the thoracic duct lymph contains both cell types. It seems that the normal 19S haemolysin

response to SRBC in the CBA mouse requires the collaboration of bone marrow-derived PFC precursors and thymus-derived "reactor cells". The neonatally-thymectomized mouse contains adequate numbers of PFC precursors but, after challenge with SRBC, few are recruited into 19S haemolysin production because of the severe deficiency in thymus-derived "reactor cells".

I. INTRODUCTION

A. The Circulating Pool of Lymphocytes and the Effects of Thymectomy on Peripheral Lymphocyte Populations

The small lymphocyte has long been recognized as the major cellular constituent of lymph and lymphoid tissues. One of the notable advances in cellular immunology over the past decade or so has been the demonstration that these morphologically identical small lymphocytes are, in point of fact, representatives of a heterogeneous population of cells with different functions and life histories. The population may be divided for convenience into three "lymphocyte compartments", namely, thymus, circulating pool and bone marrow (e.g. Miller and Osoba, 1967). As will be discussed, the bulk of the lymphocytes in these three compartments may have vastly different functions, fates, and life spans but they may be related through differentiative cell lineages. In this section the effects of thymectomy on the cellular composition of the peripheral lymphoid organs, and the circulating lymphocyte pool in particular, will be examined in detail. In addition, the possible sites of origin of the lymphocytes in organs such as the spleen and lymph nodes will be considered, the emphasis being placed on the cellular contribution of the thymus to the circulating pool.

(1) The lymph-borne lymphocytes

The technique of lymphatic cannulation has been used as a means of enumerating lymph-borne lymphocytes (reviewed in Yoffey and Courtice, 1956). Early investigations established that the lymph in the major lymphatic vessels ("central lymph") contained

many more small lymphocytes than the "peripheral lymph" which drained into organized lymphoid aggregates such as the lymph nodes and Peyer's patches (Baker, 1933; Yoffey and Drinker, 1939; Ehrich and Harris, 1942). The conclusion therefore seemed warranted that the majority of lymphocytes constantly entering the blood from the lymphatics were newly formed within the lymph nodes and were not, as suggested by Sjövall (1936), part of a recirculating population of cells migrating from blood to lymph within the connective tissues. As a necessary corollary, the lymphocytes in the blood and lymph were assumed to have a short life span and to be destined to die in some anatomical "graveyard" - a site which thwarted all attempts at identification.

The results of cell output determinations in chronically drained, unanaesthetized rats led to much revised thinking on the fate of the ubiquitous small lymphocyte. Mann and Higgins (1950) first described a progressive decrease with time in the number of cells emerging from a thoracic duct fistula in rats. With the assumption that lymphocytes were manufactured in the lymph nodes, one would not expect the number in efferent lymph to be affected by chronic drainage unless the lymph, cells, or cell breakdown products were essential for continued production of new cells within the nodes. By comparing the output of cells from the thoracic duct and the intestinal and hepatic lymphatics, Mann and Higgins also demonstrated that the major cellular contribution to the thoracic duct was from the intestinal region and presumably the mesenteric lymph nodes and Peyer's patches. This conclusion has been challenged by Fichtelius (e.g. Fichtelius, 1960; Fichtelius and Bryant, 1964) who described a preferential localization of injected lymph node and thymus lymphocytes in the liver and hepatic lymph nodes. A number of objections can be raised to these experiments and thus to the

conclusion that there is an asymmetric recirculation of lymphocytes through the hepatic lymphatics (vide infra). Mann and Higgins also found no correlation between the output of cells and the volume of lymph drained on any one day of drainage. Furthermore, rats cannulated after three days of restraint displayed no differences from rats not stressed prior to cannulation in the pattern of thoracic duct cell output.

Gowans (1957, 1959a) confirmed and greatly extended many of the observations of Mann and Higgins. Using rats maintained by reinfusion of cell-free lymph, Gowans fully documented the progressive decline in cell output over the first 4 to 5 days of thoracic duct drainage. A crucial finding was provided by Gowans (1957) when he showed that by slowly reinfusing viable thoracic duct cells, the fall in cell output could be obviated. In addition, the intravenous infusion of syngeneic lymphocytes into chronically-drained rats significantly elevated, and then maintained, the output of cells (Gowans, 1959a). The first direct evidence for recirculation of lymphocytes was provided in this paper. Thoracic duct cells were harvested from donors injected with P^{32} and were infused into other chronically-drained rats. The donor lymphocytes were shown to be labelled and the increase in radioactivity in the cells from the thoracic duct of the recipient paralleled the increased cell output. Shorter and Bollman (1960) also recorded a marked increase in the radioactivity of cells in the intestinal lymph of rats 24 hours after intravenous injections of Cr^{51}- and P^{32}-labelled thoracic duct cells. By injecting tritiated thymidine into the recipients, the increased number of cells emerging from the thoracic duct was shown by Gowans not to be due to the appearance of newly-formed lymphocytes. He concluded that constant replenishment of the blood lymphocyte

compartment was essential for maintaining the thoracic duct lymphocyte output in rats. An interesting observation, which has been adequately confirmed (Caffrey, Rieke and Everett, 1962; McGregor and Gowans, 1963; McGregor, 1966), was that the output of large and medium lymphocytes (a large proportion of which incorporate tritiated thymidine in vitro and in vivo) did not fall dramatically with increasing times of drainage. These cells were therefore most likely to have been newly formed within the lymph nodes and must differ in this respect from the majority of thoracic duct small lymphocytes.

The output of cells from the thoracic duct of unanaesthetized mice has been recorded over short periods of drainage by Shrewsbury (1959) and Boak and Woodruff (1965) and for longer periods by Gesner and Gowans (1962a), Houba and Houba (1966), Mandel (1967) and Morse and Riester (1967). As in rats, the number of lymphocytes decreased markedly some days after the commencement of drainage and the proportion of large lymphocytes increased. No correlation was observed between lymph and cell outputs and mice stressed prior to cannulation did not exhibit any alterations in the essential features of the daily cell output profile (Gesner and Gowans, 1962a).

Strong supporting evidence for the existence of a recirculating population of lymphocytes has come from the work of Hall and Morris (1964, 1965a) in sheep. Following local irradiation of the popliteal lymph node, the reduction in the number of lymphocytes in the efferent lymph was not as great as would have been expected if cells were being produced within the node. In another experiment, tritiated thymidine was infused for 100 to 150 hours into the afferent lymphatics of unstimulated nodes. The cells draining from efferent cannulae were examined autoradiographically for the presence of labelled cells. By estimating the proportion of the lymph node actually infused with tritiated thymidine, and by enumerating labelled cells in the efferent

lymph, Hall and Morris (1965a) calculated that no more than 4% of the small lymphocytes in the lymph were produced by cell division within the node. The finding that the majority of large lymphocytes had incorporated the tritiated thymidine suggested that this morphological cell type may originate in lymph nodes by processes involving DNA synthesis.

Olsen and Yoffey (1967) have called attention to the fact that the popliteal lymph node in the guinea pig is an "oligosynthetic" node. The proportion of labelled lymphocytes leaving in the efferent lymph is therefore unlikely to be as great as that from "polysynthetic" structures such as the Peyer's patches and the mesenteric and cervical lymph nodes. Even though this objection to the conclusions of Hall and Morris may influence any calculation on the extent of recirculation of lymphocytes through lymph nodes, the proportion of labelled lymphocytes leaving an infused lymph node probably reflects the degree of local antigenic stimulation (Hall and Morris, 1965a,b). Hence, the conclusion still seems valid, that in the resting node the majority of small lymphocytes in the efferent lymph have not been produced within the substance of the node.

(2) The life span of lymphocytes

That a proportion of blood and lymph-borne lymphocytes has a long life span is now firmly established. The best evidence for this comes from tritiated thymidine studies in rodents and cytogenetic studies in man (reviewed in Gowans, 1966; Elves, 1966; Everett and Tyler, 1967). Little, Brecher, Bradley and Rose (1962) and Robinson, Brecher, Lourie and Haley (1965) followed the appearance of labelled cells and, more importantly, the persistence of unlabelled cells (which were therefore undamaged by the uptake of tritium) in the

blood of rats after prolonged infusions of tritiated thymidine. The presence of unlabelled small lymphocytes after months of tritiated thymidine administration, coupled with the rapid labelling of 100% of the blood neutrophils, led these workers to conclude that some small lymphocytes in the rat may have a life span of many months if not years. Cytogenetic studies in man, using peripheral blood cells from patients exposed some time previously to therapeutic x-irradiation, have shown that, under the influence of PHA, cells with severe radiation-induced chromosomal observations can be induced into mitosis. The aberrations were considered inimical to cell viability after mitoses and it was concluded that the cells must have survived in interphase from the time of irradiation to the time of PHA stimulation. The life span of such damaged lymphocytes has been measured in terms of years (Buckton, Jacobs, Court Brown and Doll, 1962; Buckton and Pike, 1964; Court Brown, Buckton and McLean, 1965; Norman, Sasaki, Ottoman and Fingerhut, 1965). Using radioisotopically-labelled carbon and phosphorus, the life span of some lymphocytes in patients with chronic lymphatic leukaemia had previously been estimated to be greater than 12 months (Hamilton, 1954; Christensen and Ottesen, 1955). An objection to such evidence, based on the reutilization of the label and labelled DNA or DNA precursors, has been raised by Hamilton (1956).

Only a very small proportion of blood or thoracic duct small lymphocytes in a variety of species including the mouse, incorporate tritiated thymidine after short exposure times _in vivo_ or _in vitro_ (Cronkite, Bond, Fliedner and Rubini, 1959; Schooley, Bryant and Kelly, 1959; Gowans, 1959a; Everett, Rienhardt and Yoffey, 1960; Gowans, 1962; Whitelaw, 1965; Perry, Irvin and Whang, 1967). Caffrey _et al._ (1962) subjected growing rats to multiple injections of

tritiated thymidine and, by means of autoradiography, examined the peripheral blood and thoracic duct small lymphocytes at intervals thereafter. The curve of the reduction in the number of labelled small lymphocytes with time was found to be biphasic. The work of this group (summarized in Everett and Tyler, 1967) strongly suggested that circulating small lymphocytes may be considered, in broad terms, as being either long-lived or short-lived cells. Cell smears from bone marrow and thymus contained no labelled small lymphocytes 16 days after ceasing a two weeks' course of 12 tritiated thymidine injections. By contrast,the proportion of labelled small lymphocytes in thoracic duct lymph, blood, mesenteric lymph node and spleen preparations declined rapidly in the first two weeks and decreased more slowly thereafter. Estimations of the average life span of the short-lived small lymphocytes are worthless, mainly because of the problem of reutilization of label (Craddock, Nakai, Fukuta and Vanslager, 1964; Robinson et al., 1965) and the fact that the rats were still growing. On the other hand, the data provides good evidence that the great majority of small lymphocytes in the bone marrow and thymus are short lived whereas organs involved in the circulation of lymphocytes (vide infra) contain a variable number of long-lived lymphocytes. The existence of more than one population of lymphocytes in the thoracic duct lymph of rabbits was suggested by the labelling experiments of Coassin and Kline (1957) and the preponderance of short-lived lymphocytes in the thymus and bone marrow has been demonstrated by many workers (Craddock et al., 1964; Osmond and Everett, 1964; Craddock, 1965; Matsuyama, Wiadrowski and Metcalf, 1966).

In other experiments performed by the Everett group, tritiated thymidine was injected every 6 hours for 2 weeks and the rats killed at intervals during the injection regime. Plots of the percentage of

small lymphocytes labelled in different organs showed that 90 to 100% of bone marrow and thymus small lymphocytes were labelled within 4 to 5 days but that labelled small lymphocytes in spleen, blood, mesenteric lymph node and thoracic duct lymph accumulated more slowly. A sharp break was apparent in the slope of the curve of the cumulative percentage of labelled blood small lymphocytes and a far less obvious one was described in the thoracic duct small lymphocyte curve. Everett, Caffrey and Rieke (1964) and Everett and Tyler (1967) calculated from the data that 60 to 70% of the small lymphocytes in the blood and 90% of the small lymphocytes in the thoracic duct lymph were long-lived cells. These figures are in keeping with similar data and calculations reported by Rieke and Schwarz (1967) and Robinson et al. (1965). Caffrey et al. (1962) coined the term "mobilizable lymphocyte pool" (MLP) to describe the total population of lymphocytes drained from a thoracic duct fistula in two to three days. The proportion of emergent small lymphocytes, which had been labelled by multiple injections of tritiated thymidine prior to cannulation, remained constant during this period of drainage. It has been pointed out that the figure quoted for the number of cells in the MLP may be an overestimate of the number of cells actually involved in recirculation since the loss of cells from the pool may mobilise many others (Gowans, 1966). Caffrey et al. (1962), using a single rat, showed that a pulse of tritiated thymidine prior to cannulation labelled 1% of small lymphocytes but that if injected after 4 days of drainage, 10% of small lymphocytes were labelled. The absolute number of labelled small lymphocytes was unchanged and a steady state production of this cell type was therefore suggested.

(3) The route of recirculation of lymphocytes through lymphoid tissues

The experiments reported by Gowans and Knight (1964) have unequivocally established that some lymphocytes recirculate from blood to lymph and that the main site of migration from the blood stream is within the diffuse cortex of lymph nodes. In this paper the effects of stress and heparin were shown to be insignificant factors involved in cell output determinations from restrained rats receiving heparinized cell-free lymph as a reinfusion mixture. Following incubation of thoracic duct cells with tritiated adenosine or thymidine and injection into recipient rats, very few large lymphocytes were shown to be capable of migrating from blood to lymph. A large proportion of the inoculated small lymphocytes, however, appeared in the thoracic duct with no reduction, following autoradiographic processing of thoracic duct cell smears, in the mean grain count per cell. Several hours after injecting thoracic duct cell populations depleted of large lymphocytes in vitro (Gowans, 1962; Gowans and Uhr, 1966), small lymphocytes could be detected in all the lymph nodes. Initial concentrations were observed in the mid and deep cortex within the endothelial cells of the postcapillary venules and particularly in the periendothelial layers of the venules. Labelled cells were rarely present in the subcapsular sinus, lymphoid follicles and germinal centres.

Time course studies on the appearance of labelled cells in the cortex of the lymph nodes strongly suggested that the direction of migration was from blood to lymph and that cells ultimately passed out of the nodes via the medullary sinuses and, presumably, the efferent lymphatics. A partial depletion of the injected small lymphocytes could be achieved by thoracic duct drainage of the recipients. The Peyer's patches and spleen also contained many of the injected

thoracic duct small lymphocytes and the postcapillary venules in the Peyer's patches seemed to possess properties in common with similar structures in the lymph nodes. In the spleen, initial concentrations of labelled small lymphocytes were observed in the red pulp. At later time points, the Malpighian follicles and, more specifically, the areas around the central arterioles, were crowded with labelled small lymphocytes. Recent work has indicated that the cells migrate into the white pulp from the marginal zone by passing between the lining cells of the marginal sinus (Goldschneider and McGregor, 1968a).

The ultrastructural studies of Marchesi and Gowans (1964) demonstrated quite clearly that the small lymphocytes migrate from the blood into the substance of the lymph nodes by passing <u>through</u> the endothelial cells of the postcapillary venules. The lymphocytes differed in this respect from inflammatory cells, such as neutrophils and monocytes, which migrated out of the blood by passing between the endothelial cells of the venules. Hudson and Yoffey (1966) have also shown that small lymphocytes in the guinea pig bone marrow migrate between the substance of the marrow and the blood stream by passing between the lining cells of the sinusoids.

The capacity of small lymphocytes to penetrate and traverse the cells of the postcapillary venules may be related to configurations on, or constituents of, the lymphocyte surface and to certain properties of the specialized endothelial cells themselves. Gesner and Ginsburg (1964) showed that after treatment of thoracic duct lymphocytes with glycosidases, the passage of these lymphocytes through endothelial cells was largely inhibited. Trypsin also temporarily reduced the number of inoculated Cr^{51}-labelled thoracic duct cells appearing in the thoracic duct of recipient rats (Woodruff and Gesner, 1968). Goldschneider and McGregor (1968a) recently reported that by promoting lymphocyte migration in animals depleted of circulating lymphocytes,

the histological appearance of the postcapillary venule changed, the endothelial cells becoming larger and pyronin positive. Similar changes have been described by Burwell (1962) and Krüger (1968) in antigenically stimulated lymph nodes of rabbits and mice and it is tempting to postulate that this is a result of an increased migration of lymphocytes into the node. An alternative possibility is that the endothelial cells play an active role in the processing of antigen or the triggering of competent cells in the population of recirculating lymphocytes (Burwell, 1962; Ballantyne and Burwell, 1965). A further proposal, which also lacks any experimental proof, is that the endothelial cells of the postcapillary venule are plasma cell precursors and that they receive the stimulus for differentiation from migratory small lymphocytes (Sainte-Marie, 1966).

The histological consequences of chronic thoracic duct drainage have been fully documented in the rat (McGregor and Gowans, 1963; McGregor, 1966; Goldschneider and McGregor, 1968a). Immediately following prolonged periods of drainage and reinfusion of cell-free lymph, a decrease in the weight of all lymph nodes was recorded, irrespective of whether the efferent lymphatics drained into the thoracic duct (McGregor and Gowans, 1963). A marked lymphopenia was apparent after drainage and lymphocyte counts remained depressed for 3 to 4 weeks after closing the fistula. Within the lymphoid tissues, certain areas in the Peyer's patches and the cortices of the lymph nodes were depleted of lymphocytes. Lymphoid follicles and germinal centres appeared normal with respect to both number and cellularity (McGregor, 1966; Goldschneider and McGregor, 1968a). Similarly, the medullary regions of the lymph nodes displayed no differences in chronically-drained and restrained rats. In the spleen a paucity of lymphocytes was noted in the areas of the white pulp immediately surrounding the central arterioles.

It is apparent from the results of these studies, that the regions of the spleen, lymph nodes, and Peyer's patches which are depleted of lymphocytes by thoracic duct drainage are those to which intravenously-injected labelled thoracic duct lymphocytes migrate in large numbers. McGregor (1966) noted that the depleted areas of the spleen and lymph nodes often contained an increased number of large pyroninophilic cells in histological preparations but it is difficult to assess whether the absolute number of such cells was altered by chronic drainage. As mentioned previously, there is no evidence that chronic thoracic duct drainage is associated with an increase in the absolute number of newly-formed lymphocytes present in duct lymph. Atrophic changes were apparent in the thymuses of both chronically-drained and restrained rats (McGregor and Gowans, 1963) and were thus ascribed to stress effects. Thymus lymphocytes are known to be inordinately sensitive to the cytolytic effects of the steroid hormones of the adrenal cortex (Selye, 1936; Dougherty, 1952). One would expect to see changes in the thymus of cannulated and restrained animals long before the changes in the spleen and lymph nodes which have been attributed to a loss of circulating lymphocytes.

Using the technique of extracorporeal irradiation of blood, Cronkite and his collaborators documented a dramatic fall, following such a procedure, in the thoracic duct lymphocyte output in unanaesthetized calves (summarized in Cronkite, Jansen, Cottier, Rai and Sipe, 1964). A combination of extracorporeal irradiation of blood and thoracic duct drainage resulted in a severe deficiency in the number of lymphocytes in areas of the lymph nodes and spleen corresponding to the areas mentioned above in the rat. The germinal centres were reported to be intact (Cottier, Cronkite, Jansen, Rai, Singer and Sipe, 1964).

Binns and Hall (1966) have challenged the conclusion that a large scale recirculation of lymphocytes from blood to lymph occurs in all species. Various lymphatics, which in the sheep were rich in their content of lymphocytes, contained very few cells of this morphological type in the pig. However, the authors have stressed that the peculiarities of the pig lymph node, with "inverted" cortex and medulla, multiple efferent lymphatics, and the absence of medullary cords, may contribute to the paucity of lymphocytes in efferent lymphatics. It would be of interest to know whether post-capillary venules are prominent structures in the porcine lymph node.

(4) Similarities between the histological effects of thoracic duct drainage and thymectomy

An observation which is encountered in virtually all reports on the effects of thymectomy is that the number of peripheral blood lymphocytes is reduced in thymectomized animals. The existence of this deficiency was established in many early studies dating back to 1904 (reviewed in Arnason, Janković and Waksman, 1962) and has been reported in a large number of different species including the mouse and rat (reviewed in Miller and Osoba, 1967). The number of lymphocytes in the thoracic duct and circulation increases rapidly in the perinatal period (e.g. Heath, 1964) and, in general, it can be said that the effects of thymectomy on peripheral lymphocyte number are more pronounced, the earlier in life that the operation is performed. Neonatal thymectomy in the mouse was found to limit the increase in the blood lymphocyte: polymorph ratio which occurred in sham-operated mice shortly after birth. Furthermore, the leucopenia noted in some neonatally-thymectomized mice could be

attributed solely to a reduction in the number of lymphocytes (Miller, 1961, 1962a). In the experiments of Ernström and Larssön (1966), neonatal thymectomy in guinea pigs did not interfere with the postnatal increase in "circulating" lymphocytes, but a deficiency was noticed from 14 to 180 days after birth. This species difference may be related to the observation that peripheral lymphocytes are numerous at birth in species such as the guinea pig (Mandel, personal communication) and rabbit (Archer, Papermaster and Good, 1964) whereas typical lymphocytes are scarce in extra-thymic tissues of the neonatal mouse (Archer et al., 1964) and hamster (Adner, Sherman and Dameshek, 1965). Thymectomy in the young adult mouse has been followed by a progressive diminution in the number of peripheral blood lymphocytes but, when compared with the levels in intact mice of the same age, the reduction was not marked (Metcalf, 1960).

The lymphocyte deficiency following neonatal thymectomy extends to the spleen and lymph nodes and several studies have pinpointed the lesion to those areas now known to be involved in the route of recirculation of small lymphocytes. Waksman, Arnason and Janković (1962), from a histological examination of the spleen and lymph nodes of neonatally-thymectomized rats, concluded that the lymphocyte deficiency was restricted to the population of small lymphocytes in the cuffs surrounding the arterioles in the Malpighian follicles of the spleen (i.e. periarteriolar lymphocyte sheaths) and the "primary lymphoid nodules" of the lymph nodes (i.e. paracortical areas or diffuse cortex). The paucity of lymphocytes in these areas was striking, especially in view of the statement that any animal with less than 100 mgm residual thymus tissue was considered to be "thymectomized". A low or high proportion of the rats may therefore have been partially thymectomized. The photographs and the calculations

of the relative sizes of various structural entities in the spleens and lymph nodes of neonatally-thymectomized, sham-operated, and non-operated rats, clearly showed that no significant defect in the number, size, and cellularity of the lymphoid follicles or germinal centres was apparent in the tissues of any one group. Plasma cells were present in their customary position around the penicilli arterioles of the splenic red pulp and in the medullary cords of the lymph nodes. Often an increase in the number of plasma cells was seen with an associated encroachment of those areas which in non-thymectomized rats contained an abundance of small lymphocytes (Waksman et al., 1962; Azar, 1964; Schriever, Hsu and Azar, 1967).

A comparative study of the effects of chronic thoracic duct drainage and neonatal thymectomy has been conducted recently by Goldschneider and McGregor (1968a). The lymphocyte deficiency in both neonatally-thymectomized rats and rats drained of lymphocytes for 8 days, involved the splenic periarteriolar lymphocyte sheaths and diffuse cortices of the lymph nodes and Peyer's patches. Plasma cells were numerous in the medullary cords of the lymph nodes and red pulp of the spleen. The marginal zone at the boundary between red and white pulp in the spleen was prominent in neonatally-thymectomized rats and contained many reticular cells. Lymphoid follicles in the spleen and lymph nodes, and the rim of cells at the corticomedullary junction in the lymph nodes, were not obviously affected by chronic thoracic duct drainage and were histologically normal in neonatally-thymectomized rats. The photographs of Agnew (1967) indicate, nevertheless, that germinal centres may be less obvious in the lymph nodes of neonatally-thymectomized rats.

In the initial descriptions of the histological effects of neonatal thymectomy in the mouse, emphasis was placed on the

absence of all lymphoid elements in the spleen and lymph nodes including germinal centres and plasma cells (Miller, 1961, 1962a; Good, Dalmasso, Martinez, Archer, Pierce and Papermaster, 1962; Parrott and East, 1964). It now seems that germinal centres are quite normal in neonatally-thymectomized mice until the onset of wasting disease and that plasma cells may be plentiful (Parrott, de Sousa and East, 1966). As in the rat the periarteriolar lymphocyte sheaths in the spleen and the diffuse cortex of the lymph nodes of neonatally-thymectomized mice contained few small lymphocytes (Parrott, 1962; Parrott and East, 1964; Osoba, 1965a; Parrott et al., 1966; Dukor and Dietrich, 1967). These areas have been termed "thymus-dependent areas" (Parrott et al., 1966), the implication being that the cellularity of the areas, and not necessarily the functional capacity of the remaining cells, is thymus dependent.

The radiosensitivity of cells in thymus-dependent and non-thymus-dependent areas, and the repopulation of these areas in sublethally-irradiated rats, has been the subject of experiments reported by Bos (1967). Total body irradiation resulted in a rapid loss of cells from the follicles in the spleen and lymph nodes and also from the marginal zone in the outer cortex of the lymph nodes and outer regions of the Malpighian follicles. Loss of cells from the diffuse cortex of the lymph nodes and periarteriolar lymphocyte sheaths in the spleen was less obvious with the doses of irradiation employed in this study. Following local irradiation of the lymph nodes or spleen, the repopulation of the follicles and marginal zones within these organs was effected within 24 hours. On the other hand, the regeneration pattern in the thymus, following local irradiation of the organ, was different and lymphocytes were not apparent until 4 days postirradiation. Balner and Dersjant (1964) had previously shown that the slow regeneration of the lymphocyte compartment in the thymus contrasted with the thymus-independent and rapid

repopulation of lymphoid follicles in heavily-irradiated mice following an injection of labelled bone marrow cells.

Of particular significance to the present discussion are the studies reported by Bos (1967) on the histological consequences of total body sublethal irradiation combined with subsequent local irradiation of the thymus at intervals of 6 days. The thymus remained atrophic and the splenic periarteriolar lymphocyte sheaths and the diffuse cortices of the lymph nodes were virtually devoid of lymphocytes. By contrast, regenerative processes were obvious in primary follicles and marginal zones. In the converse experiment, in which rats were irradiated with the thymus shielded, the number of lymphocytes in the thymus-dependent areas was increased over that in comparable areas in the spleen and lymph nodes of totally-irradiated rats. The difficulty of shielding the thymus, without shielding associated structures such as lymph nodes and even vertebral bone marrow, may conceivably complicate the interpretations of the latter experiment (e.g. Adams, 1967). Bos, on the basis of this histological data, concluded that the follicles and marginal zones were repopulated after irradiation by blood-borne cells not derived from the thymus. Manipulations which precluded thymus regeneration seemed to specifically affect the cellular composition of these areas known to contain a large number of long-lived recirculating lymphocytes. Auerbach (1963) and Globerson and Feldman (1964) had previously shown in irradiated mice that the presence of the thymus was required for the complete regeneration of peripheral "lymphoid centres".

Several groups have reported that thymectomy does not lead to a reduction in the number of marrow lymphocytes (Bierring, 1960; Waksman et al., 1962; Bierring and Grunnet, 1964; Corsi and

Giusti, 1967a) even though a change in the relative numbers of erythroid cell types (Corsi and Giusti, 1967a) and other myeloid abnormalities (Miller, Block, Rowlands and Kind, 1965) have been recorded. Moreover, Rieke and Schwarz (1967) found no evidence for altered lymphopoiesis in the bone marrow of neonatally-thymectomized rats and Craddock, Winkelstein, Matsuyuki and Lawrence (1967b) showed that the recovery of rat marrow lymphocyte numbers after cortisol depletion was not thymus dependent. Recently, Stutman, Yunis and Good (1968) reported that the bone marrow of young adult neonatally-thymectomized mice did not lack haemopoietic colony-forming units (Till and McCulloch, 1961) even though the number of small lymphocytes was reduced by a factor of 3 when compared with the number in intact mice of the same age. The increased size of the spleen in some neonatally-thymectomized mice has been ascribed to extensive extramedullary haemopoiesis in conventionally-reared (Miller and Howard, 1964) and germ-free mice (Dukor, Miller and Sacquet, 1968). Hyperplastic changes in the reticuloendothelial system are considered to be related to infection and/or the impending onset of wasting disease (Miller, 1963; Miller and Howard, 1964; Schooley, Kelly, Dobson, Finney, Havens and Cantor, 1965).

The question of whether the reduction in the number of peripheral lymphocytes is a primary consequence of thymectomy has received considerable attention. A depletion of lymphocytes may be a direct result of the absence of a thymic influence necessary for the establishment or maintenance of peripheral lymphocyte numbers. Alternatively, it may be a secondary consequence of the heightened susceptibility of neonatally-thymectomized animals to the spectrum of microorganisms encountered in the conventional colony. These two factors may not be mutually exclusive and one may exacerbate the effects of the other. A common sequela of neonatal thymectomy is

the development of a fatal wasting disease and the condition seems to be particularly severe in conventionally-reared neonatally-thymectomized mice and hamsters in some laboratories (Miller, 1962a, 1964a; Parrott, 1962; Sherman, Adner and Dameshek, 1963). Clinical signs such as a reduction in growth rate, ruffled fur, hunched posture, inactivity and, in some animals, diarrhoea, led to the notion (Miller and Howard, 1964) that the pathogenesis of the condition was similar to the immunologically-based "runt disease" and "F_1-hybrid disease" (e.g. Billingham, 1967). On the basis of these clinical similarities and the histological appearance of tissues from neonatally-thymectomized mice, de Vries, van Putten, Balner and van Bekkum (1964) have postulated that an autoimmune process may cause, or at least contribute to, the development of wasting disease.

Early attempts to isolate pathogenic bacteria and viruses from severely wasted mice were unsuccessful (Parrott and East, 1964). In later passage experiments, in which cell-free organ extracts were injected into newborn mice, the presence of an hepatotrophic virus in the spleen and liver of some, but certainly not all, wasted mice was reported (East, Parrott, Chesterman and Pomerance, 1963). Since the virus could not be isolated invariably, East and her colleagues considered it not to be the primary cause of wasting. Young adult neonatally-thymectomized mice have been shown to be more susceptible to certain viral infections (Mori, Takeya, Minamishima and Tasaki, 1965; de Somer, Denys and Leyten, 1963), the endotoxins of some Gram negative bacteria (Salvin, Peterson and Good, 1965, c.f. Porter, Spievack and Kass, 1966), mycobacteria (Rees, 1966) and to harbour large numbers of Candida albicans cells for prolonged periods of time (Salvin et al., 1965). Miller (1964a) reported that neither high vitamin diets nor antibiotics were curative in wasting mice. By contrast, Azar (1964),

in a study in which the incidence of abscesses in surgical wounds and pneumonia in sham-operated rats was very high, concluded that tetracyclines did reduce the number of losses from wasting disease in neonatally-thymectomized rats. Similar drug therapy in animals in which wasting disease had been induced by the injection of hydrocortisone at birth (Schlesinger and Mark, 1964), significantly reduced the number of mortalities (Duhig, 1965) as did rearing the animals in a germ-free environment (Reed and Jutila, 1965, 1967).

The case for infectious agents as precipitating factors in wasting disease has been reviewed and supported recently by Miller and Osoba (1967). Perhaps the evidence which is most convincing in this regard is the demonstration that germ-free and pathogen-free mice do not display any of the signs of wasting disease (McIntire, Sell and Miller, 1964; Wilson, Sjodin and Bealmear, 1964; Hess and Stoner, 1966; Dukor et al., 1968). Such mice are, nevertheless, susceptible to induced graft-versus-host reactions (McIntire et al., 1964; Salomon and Lecourt, 1966) and, as is the case in the vast majority of conventionally-reared neonatally-thymectomized mice, rarely show histological evidence of autoimmune involvement of any organ (Dukor et al., 1968). The absence of wasting disease in germ-free neonatally-thymectomized mice, coupled with a reduction in the cellularity of the periarteriolar lymphocyte sheaths in the spleen and the diffuse cortex of the lymph nodes, has been used by Dukor et al. (1968) as evidence that a defect in the peripheral lymphocyte number is a primary consequence of neonatal thymectomy and is not related to the presence of infectious agents. The question is still open, as viruses have often been observed in tissues from germ-free mice (de Harven, 1964; Pollard, 1967) and the presence of considerable "background antigenic noise" was evident in the mice studied by Dukor et al. (1968). It is obvious, nevertheless, that

extrathymic lymphopoiesis in seemingly normal structures, such as lymphoid follicles, germinal centres and bone marrow, does not make up the defect in small lymphocytes in lymphoid organs of neonatally-thymectomized rats and mice.

(5.) Defects in the thoracic duct lymphocyte population following thymectomy

Since the bulk of the evidence points to a selective reduction of recirculating small lymphocytes in neonatally-thymectomized animals, the technique of thoracic duct cannulation should provide a means of confirming and quantitating this deficiency. Thoracic duct cell output determinations following thymectomy have been reported in the rat, mouse and guinea pig. Anaesthetized animals were used in these studies and since collection times were invariably restricted to less than two hours, the results have been expressed as the output of cells per aliquot of lymph collected or per hour of drainage. Several weeks after thymectomy in young guinea pigs, the lymphocyte output from the thoracic duct had fallen to 50% of control values (Reinhardt and Yoffey, 1956; Reinhardt, 1964; Clark, Williams and Yoffey, 1966). Bierring (1960) thymectomized young adult rats and collected thoracic duct lymphocytes two months later. The number of such cells was found to be reduced by 40% over that in controls. Similar acute drainage experiments performed by Schooley and Shrewsbury (1967) showed that thymectomy in 4 weeks old rats and mice was followed by a 50 to 60% reduction in the number of thoracic duct cells several days later. Schooley and Kelly (1964) thymectomized and sham-thymectomized 6 day old rats and demonstrated a 73% reduction in cell output in the thymectomized group 2 months later. In an abstract, Rieke (1964) reported that the thoracic duct of neonatally-thymectomized 60 - 200 gm rats contained approximately 20% of the number of lymphocytes present in the thoracic

duct of intact rats of the same body weight. The deficiency involved only the small lymphocytes, there being no difference between the two groups in the absolute number of large and medium lymphocytes.

These various studies could be criticised on the grounds that the anaesthetic agent, or other factors operating in the anaesthetised state, may have differential effects on the distribution of lymphocytes or output of lymph in thymectomized and nonthymectomized rodents. Acute output determinations in immobilized, anaesthetised animals may thus give a false picture of the effects of thymectomy on the number of cells in the circulating pool of lymphocytes.

Rieke has examined the proliferative and metabolic properties of thoracic duct cells from neonatally-thymectomized rats (Rieke, 1964, 1966). Ten to 14% of thoracic duct lymphocytes in neonatally-thymectomized rats incorporated tritiated thymidine when only 2 - 3% of lymphocytes in control rats were labelled. This difference was ascribed to the presence of increased numbers of large and medium lymphocytes in the lymph of the thymectomized group. By injecting tritiated thymidine daily for two weeks, Rieke and Schwarz (1967) found that 60% of the small lymphocytes in the thoracic duct lymph of neonatally-thymectomized rats were labelled compared with 30% of small lymphocytes in the thoracic duct of control rats. The decay in the percentage of labelled small lymphocytes was found to be biphasic in both groups and, by extrapolating the two horizontal portions of the curves, they calculated that the number of short-lived small lymphocytes was the same in both groups. The deficiency in the thoracic duct lymphocyte population in neonatally-thymectomized rats therefore seemed to be confined to the population of long-lived cells.

In another experiment, Rieke and Schwarz (1967) found that all the large lymphocytes of the blood and lymph of both thymectomized and control rats were labelled at 17 hours after the initiation of 6-hourly

injections of tritiated thymidine. These labelled large lymphocytes were reported to disappear more slowly in thymectomized animals, 10% being labelled 7 days after discontinuing a 5 day course of thymidine injections. This could quite conceivably represent an increased rate of antigen-induced blast transformation in previously labelled small lymphocytes within the environment of the neonatally-thymectomized hosts. Such immunological "cripples" (Section IC) could be expected to encounter a greater array of persisting antigenic material than rats with an intact immunological system.

Rieke (1966) incubated thoracic duct cells with tritiated uridine for 1 hour in vitro and performed grain counts in standard autoradiographic preparations of cell smears. He concluded that the thoracic duct small lymphocytes from neonatally-thymectomized rats displayed a defect in RNA metabolism since a decreased number of grains was apparent over this cell type. Twenty million lymphocytes from neonatally-thymectomized rats did not cause runt disease in newborn allogeneic recipients whereas 2 million from intact donors were invariably effective. Interestingly, the differences in the percentage of blast cells in short term PHA culture or in the percentage of mitotic figures in the presence of allogeneic or xenogeneic cells in vitro, were not as great as would have been expected from the results of the in vivo assays.

Rieke proposed that the difference in the in vitro and in vivo activity of lymphocytes from neonatally-thymectomized rats could be accounted for on the basis of a qualitative defect in these cells. From the work of Gowans (1962), small lymphocytes most probably initiate graft-versus-host reactions. The reduction in the relative number of small lymphocytes in the lymph of neonatally-thymectomized rats may necessitate the use of large cellular inocula to induce runt disease.

In addition, and more importantly, the small lymphocytes responsive to histocompatibility antigens may be diluted out by small lymphocytes committed to other antigens. There may thus be a simple quantitative defect in the thoracic duct lymphocyte population from neonatally-thymectomized rats with respect to cells competent to engage in graft-versus-host reactions. This proposition, however, begs fundamental questions about the clonal individuation and restricted reactivity of competent lymphocytes.

The finding that the difference in the activity of the two populations in vitro was not particularly striking may not be irreconcilable with the conclusions reached above. Perhaps the elaboration of factors after the initiation of blast transformation in responsive cells in vitro (e. g. Lawrence, 1967) nonspecifically recruits many others into blastoid or mitotic activity (Section IC). A further hypothesis, relevant to the status of thoracic duct lymphocytes from neonatally-thymectomized rats with respect to RNA metabolism, is most attractive in the light of the discussion to follow. Perhaps lymphocytes derived from the thymus have a higher RNA metabolic rate than lymphocytes derived from other sources. In the thoracic duct of neonatally-thymectomized rats the decreased ability of the cells to incorporate RNA precursors might then reflect a relative and absolute decrease in the number of thymus-derived lymphocytes. Thymus lymphocytes themselves incorporate less tritiated uridine than thoracic duct lymphocytes in vitro (Goldschneider and McGregor, 1968a), but the relative capacities of migrant thymus cells and nonthymus-derived cells are not known.

(6) Thymus cell migration

The finding that thymectomy affects lymphocyte numbers in the circulating pool, coupled with the well-documented and obvious

lymphopoietic activity of the thymus, has led to the notion that the lymphocytes in these two compartments may be members of the same cell lineage. If this is the case, then extirpation of the thymus simply removes the primary source of the circulating lymphocytes. By inference from the histological effects of thymectomy at different ages, the postulated peripheralization of thymic lymphocytes must be in progress very early in life and the extent of migration must decrease with increasing age.

Considerable controversy has centred around the fate of the small lymphocytes produced in the thymus and it seems that the question will be resolved only when a stable marker unique for thymus and thymus-derived lymphocytes is available. The studies of Metcalf and his co-workers, employing thymus grafting, tritiated thymidine labelling, and autoradiographic techniques, have suggested that the majority of thymus lymphocytes die within the thymus. The autonomous lymphopoietic behaviour of the thymus and thymus grafts, and the inductive influence of the epithelial cytoreticulum, were firmly established by Metcalf, Sparrow, Nakamura and Ishidate (1961) and Metcalf (1963). In mice grafted with as many as 48 thymus grafts (the weight of which amounted to 1160 mgms of autopsy), no increase in peripheral lymphocyte numbers was detected. Lymphopoiesis was, however, as intense in the grafts as in the host thymus (Matsuyama et al., 1966). By performing grain counts in autoradiographic preparations, Matsuyama et al. (1966) showed that the total amount of tritium incorporated into the thymus or thymus grafts, after a "pulse" intraperitoneal injection of tritiated thymidine, was not reduced until 3 to 4 days after the injection. A similar phenomenon was reported by Craddock et al. (1964). The total radioactivity in the thymus of rats after systemic

tritiated thymidine administration remained constant for 3 days. When considered together with the demonstration that approximately 90% of thymus lymphocytes are lightly labelled at 3 days after a single injection of H^3 thymidine, these findings suggest that total lymphocyte renewal in the thymus is rapid and that cells must be lost from the thymus environment, not on a random basis, but after 3 to 4 days.

The plateau in radioactivity may be due to a migration of labelled cells from the spleen and lymph nodes into the thymus. This possibility is rendered unlikely by the work of Gowans and Knight (1964), Galton and Reed (1966) and Goldschneider and McGregor (1968a) who showed that very few lymphocytes migrate to the intact adult thymus. In addition, very few heavily-labelled lymphocytes, of the type found in spleen and lymph nodes after pulse injections of tritiated thymidine, were found in the thymus (Craddock et al., 1964; Matsuyama et al., 1966). The possibility that bone marrow-derived lymphocytes had migrated in large numbers to the thymus does not seem to have been discounted. Such cells could be expected to be lightly labelled under the conditions of the experiments (Osmond and Everett, 1964; Craddock, 1965), and the data of Matsuyama et al. (1966) actually shows an increase in total grain counts in thymus cell smears over the 3 day "plateau" phase. Very few bone marrow cells, however, migrate to the thymus at least immediately after irradiation (Balner and Dersjant, 1964) and the results of parabiosis experiments almost exclude any possibility of massive migration of lymphocytes through the thymus (Harris, Ford, Barnes and Evans, 1964; Brumby and Metcalf, 1967).

Estimates of thymus cell migration, based on lymphocyte numbers in afferent and efferent thymic blood and lymph, have been made by Kotoni, Seiki, Yamashita and Horii (1966) in the rat and in the guinea pig

by Sainte-Marie and Kostuck (cited in Sainte-Marie and Leblond, 1964) and Ernström, Gyllensten and Larssön (1965). In the guinea pig, higher lymphocyte counts were obtained from certain thymic veins than thymic and carotid arteries and the femoral veins. Histological evidence for diapedesis of cells across the perivascular channels and the walls of medullary venules was presented by Sainte-Marie and Leblond (1964) but the direction of migration was impossible to determine. Kotani et al. (1966) described accumulations of thymocytes in septal areas traversed by lymphatic channels. They believed these channels drained to the cervical lymph nodes. By measuring the number of lymphocytes in the efferent lymph of the right cervical lymph node, Kotani et al. demonstrated that removal of the appropriate thymus lobe resulted in a 50% reduction in lymphocyte output from the node. It is difficult to assess whether the cells in septal areas of the thymus were derived from the thymic parenchyma and what effect the introduction of antigenic material during surgery might have had on the output of cells from the regional lymph node. Hall and Morris (1965b) have documented a dramatic and rapid reduction of lymphocytes in the efferent lymphatics of popliteal lymph nodes in sheep after the local application of a variety of antigens.

Intrathymic labelling studies using the labelled nucleosides, tritiated thymidine and tritiated adenosine, have been performed by Nossal (1964), Murray and Woods (1964), Weissman (1967) and Linna (1968) in an attempt to detect, and quantitate the extent of, thymus cell migration. The major criticisms of this approach relate to problems of leakage of the labelled nucleosides, reutilization of label, and damage to thymus cells. In the study of Murray and Woods (1964) tritiated thymidine was injected into one lobe of the adult guinea pig thymus. Leakage from the injection site must have occurred since lightly-labelled granulocytes

and plasma cells accumulated rapidly in various organs. The lymphocytes in the noninjected lobe were also lightly labelled even though no labelled cells were detected in the portion of the duodenal epithelial mucosa used as an indication of leakage. Nossal (1964) performed similar experiments in newborn and young adult guinea pigs. Migrant cells were distinguished from cells resident in the peripheral lymphoid tissues by a difference in the intensity of label. Small and medium lymphocytes, with 28 x the label present in labelled dividing cells of the mesenteric lymph node, were detected in small numbers within this and other nodes, particularly in those of newborn guinea pigs. The results of both these studies, and of Linna (1968) in hamsters of various ages, indicated that very few thymus cells migrated to peripheral lymphoid organs.

The recent report of Weissman (1967) has indicated that the extent of thymus cell migration in the rat may be more pronounced than had hitherto been supposed. Weissman used newborn and adult rats and, by employing a variety of controls, he seems to have performed the most satisfactory intrathymic labelling study to date. Control preparations in adult rats consisted of a certain number of adrenalectomized animals, continuous intravenous infusions of "cold" nucleoside, and the injection of labelled thoracic duct lymphocytes and bone marrow cells in an attempt to detect the infiltration of lymphocytes and inflammatory mononuclear cells into, and subsequent migration out of, the injection site. At the end of the 1.5 to 2 hours intrathymic infusion period, labelled cells were confined to the thymus and, more specifically, to the lymphocytes around the point of insertion of the needle. As might be expected, tritiated adenosine labelled all lymphocytes in the thymus, whereas tritiated thymidine was incorporated initially into large and medium lymphocytes.

The appearance of labelled small lymphocytes following tritiated thymidine injection into the thymus coincided with a migration pulse of labelled cells in peripheral lymphoid organs. In adult rats, the migrant cells were confined to the diffuse cortex of the lymph nodes and the periarteriolar lymphocyte sheaths of the spleen. Small - and medium-sized migrant lymphocytes were observed in adults and all three morphological cell types, large, medium and small lymphocytes, appeared to migrate from the thymus in newborn animals. Control preparations were not, however, applied as rigorously in newborns as in adults. By calculating the proportion of thymus lymphocytes labelled and by enumerating migrant cells in the diffuse cortices and periarteriolar sheaths, Weissman concluded that cell migration from the adult thymus could renew the entire circulating lymphocyte pool in 1 to 2 months. In the newborn a case could be made out for the hypothesis that virtually all of the lymphocytes in the spleen and lymph nodes were derived from the thymus. Weissman assumed that the majority of thymus cell migrants persisted in peripheral lymphoid tissues.

If, as Weissman suggests, the circulating pool is being replaced every 1 to 2 months in the adult, one is faced with the problem of accounting for the slight effects of adult thymectomy on the number of circulating lymphocytes. Decreased, rather than increased, extrathymic lymphopoiesis has been recorded in the lymphoid organs of adult-thymectomized mice (Metcalf, 1960; Metcalf and Brumby, 1966; Metcalf, 1967). Such a mechanism is thus unlikely to be in operation to compensate for the deficit caused by the elimination of the major source of the circulating lymphocytes. A possible explanation is that thymus lymphocytes do migrate in large numbers but have a short life span within peripheral

lymphoid organs unless required in the participation of immunological responses. Perhaps these few persisting migrants are selected for, or altered by, antigen and they or their progeny then enter the circulating pool of lymphocytes. In the adult rat the majority of cells in the circulating pool may carry immunological memory (Section IB) and are certainly long lived. Hence, the requirement for extra cells from the adult thymus is presumably minimal.

A non-immunological, trephocytic function of lymphocytes would resolve this dilemna (Loutit, 1962; Rieke, 1962; Bryant, 1963; Fichtelius and Bryant, 1964; Fichtelius and Linna, 1967). Thymus lymphocytes would simply die and furnish nuclear building blocks for other cell types be they lymphocytes, parenchymal cells of various organs, or cells involved in wound repair. This function of lymphocytes is difficult to prove; trophic effects cannot be attributed to lymphocytes only (Robinson and Brecher, 1963) and the presence of the thymus or thymus grafts does not appear to influence kidney regeneration or erythropoiesis (Metcalf, 1964). Linna (1967) was unable to demonstrate any transfer of H^3-labelled material from intrathymically-labelled thymus cells to regenerating liver cells in the guinea pig. At variance with this finding, Fichtelius and Bryant (1964) and Craddock, Rytömaa and Nakai (1964) have presented evidence that the regenerating liver may reutilise labelled DNA or DNA precursors liberated from labelled leucocytes and thymocytes. It has yet to be resolved whether lymphocytes, and in particular, thymus-derived lymphocytes, have no role to play in the body's economy other than the provision of nucleoprotein, amino acids, histones, and nucleic acids for reutilization by other cells. The innumerable demonstrations of immunological activity

of purified populations of small lymphocytes in adoptive transfer systems militates against the concept of a simple trophic function for this morphological cell type (Section IB and IC).

Evidence for migration of lymphocytes from chromosomally-marked thymus grafts in neonatally-thymectomized and in adult-thymectomized, irradiated and marrow protected mice has come from the work of Miller (1962b), Harris and Ford (1964), Miller, Osoba and Dukor (1965), Leuchars, Cross, Davies and Wallis (1964), Leuchars, Cross and Dukor (1965), Dukor, Miller, House and Allman (1965), Davies, Leuchars, Wallis and Koller (1966), Ford (1966a), Leuchars, Morgan, Davies and Wallis (1967) and Davies, Festenstein, Leuchars, Wallis and Doenhoff (1968a). Dividing graft-derived cells were detected in the spleen and lymph nodes and an increase in the number of such cells occurred after antigenic stimulation (Leuchars et al., 1964; Davies et al., 1966; Miller, de Burgh, Dukor, Grant, Allman and House, 1966). Davies et al. (1968a) used phytohaemagglutinin to stimulate peripheral blood lymphocytes from adult-thymectomized mice which had been irradiated, bone marrow inoculated, and thymus grafted up to 50 days previously. They demonstrated that approximately 80% of the mitotic figures carried the chromosome marker of the thymus graft. If thymus-derived cells are more resistant to in vitro culture conditions, or are preferentially stimulated by PHA (Section IC), then this figure may be a gross overestimate of the proportion of total peripheral blood lymphocytes which are in fact derived from the thymus graft.

The value of this data to the question of thymus cell migration from the intact thymus in situ is debatable. As has often been emphasised (e.g. Matsuyama et al., 1966; Miller and Osoba, 1967), the process of grafting leads to disruption of the thymus capsule and the release of thymocytes. When the architecture of the graft is restored, the lymphocytes, which after two or three weeks may all be host in

type (Dubert and Kaplan, 1961; Green, 1964; Metcalf and Wakonig-Vaartaja, 1964; Dukor et al., 1965; Ford, 1966a; Schlesinger and Hurvitz, 1968), may not migrate in large numbers. This receives some support from the data of Davies et al. (1966, 1968a) which shows a surprisingly large proportion of thymus graft-derived cells in the peripheral lymphoid organs some weeks after grafting. If thymus cells migrate extensively then this number would be expected to be diluted out by host type mitotic figures rather rapidly. Perhaps the lymphopenia following irradiation is much reduced at the time of complete thymus graft repopulation and the call for lymphocytes in peripheral tissues is less. This is unlikely to be so, however, as the recovery of circulating and peripheral blood lymphocyte numbers following whole body irradiation is slow and is noticeable only after two weeks (Ross, Furth and Bigelow, 1952; Hulse, 1963). In adult thymectomized mice, which are not lymphopenic, thymus graft-derived cells were detected in small numbers in the lymph nodes and spleen and no increase in their number occurred after sheep erythrocyte challenge (Leuchars et al., 1967). This may reflect either a lack of thymus cell migration or a massive dilution of migrants by long-lived lymphocytes present in the host at the time of thymus grafting.

The fate of intravenously-injected thymus cells has been studied using P^{32}-, H^{3}-, and acridine-labelled cells (reviewed in Fichtelius, 1960; Fichtelius and Bryant, 1964; Elves, 1966). This whole experimental approach as an indication of thymus cell migration and thymus cell function has been criticised on many grounds (Lajtha, 1960; Gowans, 1959b; Elves, 1966). The apparent organ localization of the cells may depend on the viability of the inoculated cells, the infusion rate and, unless a high proportion of the cells are labelled and the label is relatively stable, the results obtained may be quite fallacious. Furthermore, the injection of cells from a disrupted

thymus into the blood stream of recipient animals can hardly be considered remotely physiological (Gowans, 1959b; Fichtelius, 1960). In homotransfusion experiments, Fichtelius and co-workers (e.g. Fichtelius, 1960; Fichtelius and Bryant, 1964) traced cells to many organs but to the spleen and liver in particular. Murray and Murray (1961) found a preferential localization of thymus cells in the spleen of irradiated rats and Mims (1962) and Everett et al. (1964) also singled out the spleen of mice and rats as being the organ to which infused H^3-labelled thymus cells homed in large numbers. Everett et al. (1964) detected a small number of labelled cells in the thoracic duct lymph within 4 hours of thymus cell injection. Both Everett (1964) and Gowans (1964) have stated, however, that thymus cell inocula do not boost the thoracic duct lymphocyte output in draining rats. Moreover, Goldschneider and McGregor (1968b) recently demonstrated that injected thymocytes failed to elevate the thoracic duct lymphocyte output whereas equal numbers of thoracic duct lymphocytes were highly effective.

It can be said that the results of experiments on the fate of inoculated thymus lymphocytes can not be used as evidence that the thymus normally contributes cells to the population of thoracic duct lymphocytes. The experiments simply indicate that a small number of lymphocytes resident in the thymus at any one time can be shown to be capable of passing from blood into the thoracic duct lymph. Transfusion experiments did, however, lead Fichtelius to postulate as early as 1953 that the thymus exports lymphocytes to the spleen in which location they are able to participate in immunological reactions, possibly by transforming to plasma cells (Fichtelius, 1960)

(7) Non-thymic origin of circulating pool lymphocytes

If it is assumed that peripheral circulating pool lymphocytes are the end products of lymphopoiesis in "central" lymphoid organs then, an organ having a function akin to the avian bursa of Fabricius (Section IC) should be a source of peripheral lymphocytes in the mammal. Conceivably, production of lymphocytes could be initiated in such an organ which later in life may not bear any resemblance to an intensively lymphopoietic organ such as the thymus. The population of lymphocytes originally derived from this site might then be maintained by replication of the lymphocytes themselves. Evidence for the expansion of peripheral lymphocyte populations comes from the work of the Gowans group and will be discussed in Section IB. Replication of lymphocytes in this manner appears to be antigen dependent and a paucity of lymphocytes in the blood, lymph nodes, and spleen of germ-free mice, rats and chickens strongly suggests that, in the absence of numerous antigenic insults, the circulating pool of lymphocytes may be strikingly reduced in size (Thorbecke, Gordon, Wostman, Wagner and Reyniers, 1957; Gordon, 1959; Wilson, Bealmear and Sobonya, 1965; Olson and Wostmann, 1966; Dukor et al., 1968).

Structures which qualify on lymphopoietic criteria as candidates for the site of origin of peripheral lymphocytes are the germinal centres and the bone marrow. From the autoradiographic data of Fliedner, Kesse, Cronkite and Robertson (1964), lymphopoiesis in the germinal centres does not result in the export of large numbers of lymphocytes. In support of this, histologically normal follicles and germinal centres have been reported in the presence of a severe deficiency of lymphocytes in the spleen, lymph nodes and peripheral blood (vide supra).

Goldschneider and McGregor (1966) have produced evidence that both the thymus and bone marrow can contribute cells to the thoracic duct lymphocyte population. A certain, albeit unknown, proportion of the thoracic duct lymphocytes from F_1 rats, injected at birth with either parental type bone marrow or thymus cells, possessed the immunological characteristics of the parental cells. Thoracic duct lymphocytes from adult (Lewis x DA)F_1 rats, inoculated at birth with 50 million Lewis bone marrow or 100 million Lewis thymus cells, were transferred to irradiated (Lewis x BN)F_1 rats presensitized against DA histocompatibility antigens. An inoculum of 100 million thoracic duct cells (calculated to contain 1 million parental type cells) killed a large number of the secondary hosts in contrast to the negligible graft-versus-host activity of an equal number of thoracic duct lymphocytes from uninjected (Lewis x DA)F_1 rats. Inocula of 50 million Lewis bone marrow cells and 100 million Lewis thymus cells were themselves unable to kill many of the secondary hosts. Such an experimental system cannot be expected to yield any information on the proportion of thoracic duct lymphocytes which are bone marrow derived or thymus derived or whether the bone marrow precursors have been influenced by the thymus prior to, or during, their maturation into immunocompetent cells.

In summary, two conclusions arising from the results of experiments discussed in this section seem to be well founded. Firstly, the majority of thoracic duct small lymphocytes, in the rat at least, are long-lived cells which continuously migrate from blood to lymph within the lymph nodes by passing through the postcapillary venules of the diffuse cortex. The results of thoracic duct output determinations in several other species, coupled with the evidence for a long life span of many peripheral blood and lymph-borne lymphocytes, indicate that

large-scale recirculation of lymphocytes may be common to many species. Such a phenomenon would readily account for the fate of the large number of lymphocytes discharged into the blood from the major lymphatics each day.

The second general conclusion is that a paucity of lymphocytes in peripheral lymphoid organs is almost an invariable histological consequence of thymectomy. Recent evidence suggests that the lymphocyte deficiency, which may be exacerbated by intercurrent infections and which does not extend to entities such as lymphoid follicles, germinal centres and plasma cells, may be confined to the population of recirculating, long-lived small lymphocytes. Intrathymic labelling studies indicate that a proportion of thymus lymphocytes are destined to leave the organ but the extent of cell migration to peripheral lymphoid organs is unknown. It remains to be determined whether emigrant thymus cells are potentially long-lived small lymphocytes invested with the capacity to enter the recirculating pool directly. Thymus cell migrants could quite conceivably be short-lived cells. The hypothesis that the thymus is the principal source of recirculating lymphocytes remains attractive but no quantitative data is available to indicate whether a majority or a minority of thoracic duct lymphocytes are thymus derived.

B. The Concept of the Antigen-Reactive Cell

The nature of the cellular events of various immunological responses has occupied the attention of many immunologists for some time. As a sequel to the interest in the identity of the cell type actively engaged in antibody production, the focus of attention has now centred on the precursor cell which initiates the immune response. The immunologically competent cell has been defined by Medawar (1963) as a cell which, whilst not yet engaged in an immune response, has the capacity of doing so when stimulated by antigen. The cell is therefore endowed with the property of antigenic reactivity and can only be envisaged in retrospect since it is required to demonstrate its activity before qualifying for the title of immunologically competent or immunocompetent cell. The term has, in general, been restricted to cells responsive to foreign histocompatibility antigens and thus to cells involved in the initiation of transplantation immune reactions (Wilson and Billingham, 1967).

In those reactions in which the measurable consequence of antigen injection is the production of specific antibody molecules, the equivalent cell type has recently been termed the antigen-sensitive, antigen-target, antigen-responsive, or antigen-reactive cell. This postulated cell corresponds to the X cell in the scheme of immunogenesis proposed by Sercarz and Coons (1962) and Nossal (1965) and to the PC_2 and also the PC_1 cell of the Makinodan group (e.g. Makinodan and Albright, 1963). The antigen-reactive cell can be considered as a cell type which responds to antigen either by producing antibody or by producing a progeny of antibody-forming cells. The morphological identity of this precursor cell could be any one or several of the multitude of cell types resident in, or migrating through, lymphoid tissues. Possible candidates include macrophages, fixed primitive reticular cells, large, medium or small lymphocytes, large pyroninophilic cells or even plasma cells and their differentiating precursors.

(1) Generation of plasma cells from their precursors

The antibody producing capacity of the plasma cell is now well established and quite undisputed (Fagraeus, 1948; Leduc, Coons and Connolly, 1955; Nossal and Mäkelä, 1962; Baney, Vazquez and Dixon, 1962). Experiments employing radioactively-labelled DNA precursors have unequivocally demonstrated that the vast majority of mature plasma cells, in situations in which maximal production of such cells is evident, are end cells and the result of recent mitotic divisions (Schooley, 1961; Nossal and Mäkelä, 1962; Baney et al., 1962; Urso and Makinodan, 1963). As mentioned earlier, the identity of the precursor of this expanding population of plasma cells has been the subject of much debate. According to the concepts of Burnet (1959), this ancestral cell would be expected to be genetically restricted in its reactivity and to respond to antigen by proliferation. The end result of this proliferation would be a clone of antibody-producing cells, each elaborating antibody molecules specific for the antigenic determinant which elicited the clonal expansion. Depending on the morphological identity and the antibody-producing status of the precursor cell, this proliferative response may or may not be associated with differentiation.

On the basis of the results of pulse labelling experiments, Nossal and Mäkelä (1962) concluded that, in the secondary response to Salmonella adelaide flagella antigens in the rat, the precursor of the antibody-producing plasmablasts and mature plasma cells was a cell type which incorporated tritiated thymidine prior to antigenic challenge. The same conclusion seemed to apply to the cellular events of the primary immune response to flagella antigens (Nossal, Mitchell and McDonald, 1963). The antigen-reactive cell in this system may therefore be a DNA-synthesizing member of a "proliferating" population of cells. The possibility that reutilization of the label

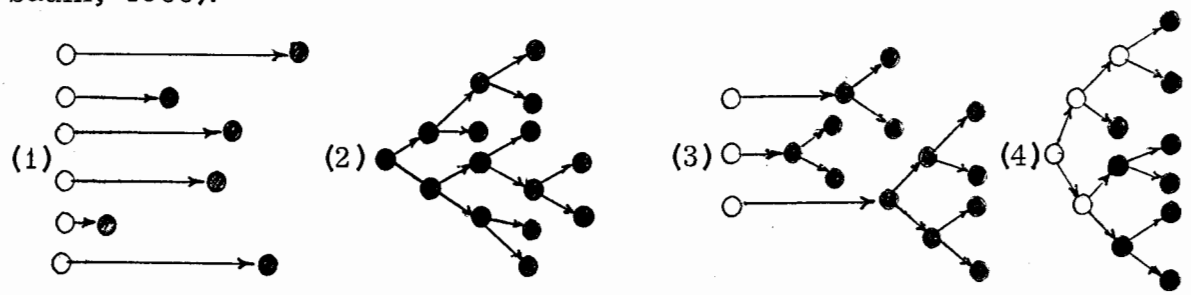
(Mitchell, McDonald and Nossal, 1963; Robinson et al., 1965) accounted for the high number of labelled plasma cells in the stimulated nodes could not be dismissed, even though it was stated that the pattern of labelling in the early nonantibody-producing blast cells did not lend support to this contention (Nossal et al., 1963). On this latter point it can not be known with certainty whether the heavily-labelled blast cells were in fact the precursors of antibody-producing cells. They may have been short-lived cells which were destined to die soon after antigen injection. Any labelled material liberated into the milieu of the node would presumably be available for incorporation into the cells of the proliferating and differentiating plasma cell line.

Cohen and Talmage (1965) used a transfer system in which spleen cells from mice stimulated with bovine serum albumin were incubated in vitro with tritiated thymidine and were subsequently injected into syngeneic irradiated recipients. They found that a proportion of anti-BSA antibody-forming cells (identified by the indirect fluorescent antibody technique) were labelled only when spleen cells were incubated 6 to 24 hours after the donor mice were challenged with antigen. Since no antibody-producing cells were labelled when spleen cells were removed immediately after challenge, these workers concluded that the precursor cell was a non-cycling cell type which entered S phase, by commencing DNA synthesis, some time after antigenic stimulation. They therefore suggested that reutilization of label accounted for the large number of labelled plasma cells in the experiments of the Nossal group. It is possible of course that certain antigens may temporarily suppress the incorporation of DNA precursors into antigen-reactive cells which are indeed members of a proliferating, or at least a cycling, cell population. This arrest may last for 6 hours in which case the results of Cohen and Talmage might be anticipated.

(2) Generation of haemolysin-forming cells from their precursors

Two techniques, capable of enumerating single antibody-producing cells in the presence of a large excess of non-antibody producers, have provided much quantitative information on the immune response to heterologous erythrocyte antigens and have added a new dimension to studies of the cellular dynamics of antibody production. The plaque-forming cell (PFC) assay, described independently by Jerne (Jerne, Nordin and Henry, 1963; Jerne and Nordin, 1963) and Ingraham and Bussard (1964), detects haemolysin-releasing cells by virtue of the appearance of pinpoint areas of haemolysis in nutrient gels containing the appropriate erythrocytes mixed with cells obtained from immunized animals. Erythrocytes immediately surrounding a single antibody-producing cell within the semisolid gel are sensitized and lysed after incubation at 37°C and the addition of complement. Following the injection of sheep erythrocytes into mice, the number of PFC in the spleen increases from a "background" number of about 100 at the time of immunization to levels approaching 100,000 4 to 5 days later (Jerne et al., 1963; Friedman, 1964; Miller, de Burgh, and Grant, 1965; Wortis, Taylor and Dresser, 1966; Zaalberg, van der Meul, van Twisk, 1966; Wigzell, Möller and Andersson, 1966; Berenbaum, 1966; Hege and Cole, 1966; Eidinger and Pross, 1967). The increasing number of such cells in the exponential phase of the response could be (1) the result of non-mitotic differentiation of as many non-antibody-producing precursor cells, these cells commencing antibody synthesis at various times after antigenic challenge, (2) the consequence of a series of cell divisions, subsequent to antigenic stimulation, on the part of a limited number of antibody-producing precursor cells, (3) a combination of the 2 previous schemes, or (4) the result of proliferation of a small number of nonantibody-producing precursor cells with differentiation to antibody-secreting cells subsequent to, or during the course of, the proliferative response.

These four models may be illustrated, in their simplest form, in the following manner where O is a cell not producing antibody and ● is an antibody-secreting cell (extension of the illustrations in Berenbaum, 1966):



Syeklocha, Siminovitch, Till and McCulloch (1966), using the drug vinblastine (VLB), have demonstrated that the precursors of PFC are not dividing as rapidly in the absence of antigen as in its presence. VLB at certain dose levels is known to cause an irreversible loss of the capacity for cell proliferation. In the experiments of Syeklocha et al., doses of VLB in excess of 0.1 mgm per mouse resulted in a dose dependent reduction of up to 1,000 fold in the number of PFC in the spleen 3 to 4 days after an injection of sheep erythrocytes. If antigen initiates a change in the growth kinetics of precursor cells, then injecting VLB before antigen should have a less dramatic effect on the responsiveness of mice to sheep erythrocytes. By injecting 0.3 mgm VLB 3 days prior to antigen (a protocol which supposedly ensured that very little VLB remained at the time of immunization), a depression in the number of PFC of less than a factor of 2 was obtained. Even with highly lethal doses of VLB and the use of an adoptive transfer system in irradiated mice, the deficiency in the response to sheep erythrocytes was by no means as great as when smaller doses of the drug were injected after antigen. The data supports the proposition that precursor cells generate PFC by processes involving cell division and that these cells are dividing far

more rapidly after immunization than immediately prior to antigen administration.

Work in the same laboratory by Kennedy, Till, Siminovitch and McCulloch (1965b) further established that the radiation sensitivity of the primary immune response of mice to sheep erythrocytes was of the same order of magnitude as that for mammalian cell proliferation in general. Groups of mice were given graded doses of total body irradiation of up to 700 rads followed by sheep erythrocytes within 2 hours of irradiation. Doses of antigen and the time of PFC assay following irradiation were varied, and, on this basis, the study was more satisfactory than that of Uyeki (1967) addressed to the same question. By constructing semi-log survival curves of PFC numbers relative to those in unirradiated control mice, the D_{37} was found to be 80 rads and was thus very similar to values obtained in other related immunological systems in which antibody titres were measured (Makinodan, Kastenbaum and Peterson, 1962; Celada and Carter, 1962). The only mammalian cell function with such a high radiation sensitivity is believed to be the capacity for continued proliferation (e.g. Kaplan, 1966). In marked contradistinction to the radiation sensitivity of the cellular system responsible for the generation of PFC, the ability of cells to release haemolysing antibodies was not affected by < 2,000 rads *in vitro* or *in vivo* and many cells retained the capacity to release antibody after as much as 9 kilorads (Kennedy *et al.*, 1965b).

Berenbaum (1966) and Rowley, Fitch, Mosier, Salliday, Coppleson and Brown (1968) used various drugs to inhibit the *in vivo* PFC response of rats and mice, and the *in vitro* PFC response of mouse spleen cells, to sheep erythrocytes. The drugs used, namely thioguanine, colchicine and VLB, are believed to be immunosuppressive by virtue of their effects on the mitotic activity of cells (Schwartz, 1965).

Dutton and Mishell (1967a, b) used the "hot pulse" technique to assess the role of cell proliferation in the PFC response of normal mouse spleen cells to heterologous erythrocytes. In their in vitro system, the incorporation of "hot" tritiated thymidine inhibited the response, and was presumably lethal to cells, only when added to the culture after 24 hours. They suggested from this evidence that DNA synthesis was minimal in the first 24 hours of culture and that all PFC arose by cell division. With two different heterologous erythrocytes and the judicious use of "hot" tritiated thymidine, Dutton and Mishell showed that inhibition of the response to one antigen did not affect the PFC response to the other. Hence, the proliferative response was associated with a high degree of specificity with respect to antibody formation. As first reported by Bussard (Bussard, 1966; Bussard and Lurie, 1967), the peculiarities of the behaviour of peritoneal exudate cells in vitro were also noticed in the system of Dutton and Mishell (1967b). Mitotic inhibitors and "hot" tritiated thymidine did not seem to affect the generation of PFC from sheep erythrocyte-stimulated peritoneal exudate cells in vitro.

These various studies emphasise the importance of proliferation in the generation of PFC in the spleen of the mouse and rat. Cell proliferation has long been considered a concomitant cellular event in antibody production and, within stimulated lymphoid organs, is apparent in both antibody-producing and nonantibody-producing cells (Wissler, Fitch, La Via, Gunderson, 1957; Schooley, 1961; Nossal and Mäkelä, 1962; Baney et al., 1962; Balfour, Cooper and Meek, 1965). Wortis et al. (1966) measured the increase in spleen cell number after a challenge injection of sheep red cells in mice. They calculated that only 1% of the increase could be accounted for by both 19S and 7S haemolysin-forming cells. The increase in spleen cell numbers could reflect recruitment of cells into the spleen and/or a burst of cell

proliferation. The demonstration that measures which are inimical to cellular proliferation decrease the number of PFC arising in the spleen after sheep erythrocyte injection, does not prove that PFC have arisen by proliferation from precursor cells. There is little doubt that PFC in mitosis are commonly encountered (Claflin and Smithies, 1967; Jerne, 1967a; Nossal, Cunningham, Mitchell and Miller, 1968) but it is still possible that proliferation may be an essential property of another cell population, this proliferative response ensuring that precursors differentiate into PFC.

Direct differentiation of precursor cells to PFC has been invoked to explain, in part, the explosive increases in PFC in the spleen between days 2 and 4 after sheep erythrocyte challenge (Sterzl, Vaselý, Jílek and Mandel, 1964; Sterzl and Jílek, 1967; Malaviya and Tannenberg, 1967; Eidinger and Pross, 1967). The doubling time of PFC in the exponential phase, bearing in mind the proposed 12 - 18 hour generation time for mammalian cells (Defendi and Manson, 1963), does seem to be too rapid to be accounted for by division of PFC alone. This as a conceptual difficulty has been challenged recently by Rowley et al.(1968) who cite the data of Metcalf (1966), Wagner, Cunningham, Cottier, Jansen, Rai and Cronkite (1967) and Saffier, Cottier, Cronkite, Jansen, Rai and Wagner (1967) to point out that lymphoid cells may have very short cell cycle times.

The observation that few PFC were labelled after tritiated thymidine or C^{14} thymidine injections and infusions appears to support the existence of mechanisms of cellular reactivation and transformation, rather than proliferation, in the evolution of PFC (Sterzl et al., 1964; Tannenberg, 1967). In addition, Sterzl (1961) reported that the 19S phase of the immune response was not affected by inhibition of DNA synthesis. The positive findings of a large number of labelled PFC in rats and mice responding to sheep erythrocytes is of much greater

significance than the negative results mentioned above (Ellis, Gowans and Howard, 1967; Szenberg and Cunningham, 1968). The PFC in these two latter systems could have acquired label from a DNA-synthesizing nonantibody-producing precursor cell or they may have incorporated the label after entering a DNA synthetic phase of the cell cycle subsequent to producing antibody. A recent report has indicated that a large number of PFC do incorporate H^3 thymidine in short term in vitro cultures (Koros, Mazur and Mowery, 1968).

Relatively large numbers of unlabelled PFC were observed by Ellis et al. (1967) and Szenberg and Cunningham (1968) in the early phases of the response to sheep erythrocytes. Ellis et al. suggested that this would be expected if "early" PFC were derived from a non-dividing precursor cell. It must be borne in mind, however, that interpretations are complicated by the presence of "background" PFC in uninjected animals. Very few "background" PFC incorporate H^3 thymidine (Szenberg and Cunningham, 1968) and if the relative contribution of these cells to the total number of PFC during, or soon after, the latent period is great, one might expect not to see many labelled PFC at early sampling times. The role of the "background" PFC in the response of animals to sheep erythrocytes will be discussed later.

(3) The number of antigen-reactive cells in the mouse spleen

The work of two groups in particular has suggested that the normal mouse spleen contains a limited number of cells which respond to sheep erythrocytes by proliferation and differentiation into PFC. Kennedy, Siminovitch, Till and McCulloch (1965a), Kennedy, Till, Siminovitch and McCulloch (1966), Playfair, Papermaster and Cole (1965) and Papermaster (1967) described the appearance of discrete foci of haemolysis in the spleens of lethally-irradiated mice injected with lymphoid cells and sheep erythrocytes. At various times after

irradiation, cell injection,and sheep erythrocytes challenge, spleens were cut into slices which were in turn laid in sequential order on regular Jerne agar plates containing sheep erythrocytes. After incubation of the plates and addition of complement, zones of haemolysis or "active areas" appeared in the agar-erythrocyte layer in association with some of the slices or portions thereof. These foci, which were noticed to be larger than the plaques resulting from the activity of a single PFC in agar, were presumed to indicate the sites of clusters of PFC in the spleens of the irradiated mice. A restricted distribution of PFC in spleens of mice was evident also in the work of Celada and Wigzell (1966) and Nakano and Braun (1966). Kind and Campbell (1968) showed that positive slices in the assay system of Playfair et al. (1965) contained more PFC than negative slices.

A linear relationship was found to exist between the number of nucleated cells injected and the number of foci appearing in excess of the "background" number, the line of best fit extrapolating to zero (Kennedy et al., 1965a). The number of foci in the spleens of irradiated mice injected with erythrocytes only, nucleated cells only, or in spleens of mice which were left uninjected, was considered to be the "background" number and was subtracted from all the experimental results. When the spleens from some mice were assayed for PFC and others for haemolytic foci, the number of foci was found to be constant between days 7 and 10 after irradiation whereas the number of PFC increased exponentially and then decreased in this time span of 4 days (Kennedy et al., 1965a, 1966). The mean number of PFC per focus at the peak of the response was calculated to be 46 following the injection of sheep erythrocytes and a fixed number of spleen cells. Lymph node cell inocula, mixed with foetal liver cells, gave rise to foci calculated to contain a mean number of 23 PFC at the peak of the response. The hypothesis was advanced that each focus resulted from the clonal proliferation and differentiation, through several generations, of an antigen-

sensitive "founder" cell which had localized in the spleen. Additional evidence that the foci resulted from a limited number of single entities was provided by Kennedy et al. (1966) using a limiting dilution assay. This particular assay was also used to substantiate the calculations now to be described, on the number of antigen-reactive cells in the normal mouse spleen.

The focus assay has been used by Kennedy et al. (1966) and Playfair et al. (1965) in experiments designed to estimate the total number of antigen-reactive cells in any one population of lymphoid cells. Meaningful numbers of mice were used by the Kennedy group in a double transplantation technique in which irradiated mice injected with spleen cells were themselves used as donors of cells for passage into further irradiated mice. The method is similar to that described by Siminovitch, McCulloch and Till (1963) for determining the fraction of colony forming units which localise in the spleen of irradiated mice, there to produce macroscopic haemopoietic colonies. One group of irradiated mice was injected with a certain number of spleen cells (= x) and sheep erythrocytes and the spleens assayed for their content of haemolytic foci 8 days later (=y). Other mice were injected with 10x spleen cells and single cell suspensions were prepared from the spleens of these mice 2 hours after cell injection. Further irradiated mice were injected with 10x spleen cells from these hosts together with sheep erythrocytes. The number of foci was determined 8 days later (= z). The fraction (f) of focus-producing, or antigen-reactive cells, localizing in the spleen is thus $y/10z$. This fraction was found to have a mean value of 15% and Kennedy et al. (1966) used this figure to calculate that the normal mouse spleen contained approximately 1000 antigen-reactive cells.

Using a similar transplantation technique, but very few assay mice, Playfair et al. (1965) calculated the number of antigen-reactive cells to be 5000 per spleen. Papermaster (1967), using a radioisotope

technique, subsequently reduced this figure to 2000. In these latter experiments, irradiated mice were injected with I^{125}- or Cr^{51}-labelled spleen cells and the percentage of injected radioactivity in the spleen determined 24 hours later. The percentage of recoverable counts in recipient spleens varied from circa 5 to 15. Gregory and Lajtha (1968) also estimated f to be approximately 10%.

As pointed out by Jerne (1967b), the figure of 10% for the localization of antigen-reactive cells into the irradiated spleen, may give estimates of the total number of antigen-reactive cells in lymphoid inocula which are higher than the actual number. The transfer methods do not take into account further recruitment of antigen-reactive cells into the irradiated spleen after 2 - 24 hours. The f value cited may therefore be incorrect by an order of magnitude butit is of interest to note that the figure of 1000 antigen-reactive cells per spleen correlates well with that obtained using a radiobiological assay (vide infra). It is higher than the figure mentioned by Makinodan and Albright (1967) and Jerne et al. (1963) by a factor of 1 $\log_{10}$. Jerne et al. assumed symmetrical divisions of PFC over a number of generations and, from the rate of increase in PFC number in the spleens of normal mice, estimated the frequency of target cells to be of the order of 10^{-6} for sheep erythrocytes antigens. The conclusion that the increase in PFC numbers is too rapid to be accounted for simply by proliferation of PFC has been discussed previously and has been supported recently by Jerne (1967a).

Makinodan and Albright (1967) used a limiting dilution assay with rat erythrocytes and spleen cells in in vivo diffusion chambers and arrived at a similar figure. These determinations, based on antibody titres, are open to objections such as the unknown sensitivity of the antibody titrations and the extent of cell death in diffusion chambers.

Even if the figure for the number of antigen-reactive cells per spleen was known with certainty, it would be unwise to use it in calculations of the minimum number of generations involved in the production of PFC from their precursors. The percent recruitment of available antigen-reactive cells would still be an unknown quantity. Moreover, the work of Ford (Ford, Gowans and McCullagh, 1966; Ford and Gowans, 1967) has clearly shown that the magnitude of the haemolysin response of the isolated and perfused rat spleen is determined more by the number of cells (in this case lymphocytes) migrating through the organ rather than the resident number.

The experiments of Kennedy et al. (1965b) on the radiosensitivity of the haemolysin response to sheep erythrocytes also give some indication of the total number of antigen-reactive cells present in the spleens of normal mice. Following 700 rads γ-irradiation the plaque-forming cell response to sheep erythrocytes was reduced by a factor of 1000. Nevertheless, the response in the spleen (about 100 PFC) was greater than the background number of plaque-forming cells in the spleens of heavily-irradiated mice not injected with sheep erythrocytes. Implicit in the argument formulated by these workers is that the effects of γ-irradiation are "all or none" and that some cells will escape with their proliferative capacities unaffected. The 100 or so PFC appearing in response to sheep erythrocytes in the spleens of mice receiving 700 rads may therefore be the outcome of at least 6 - 7 generations of cells in a clone which originated from one antigen-reactive cell. In this case the number of antigen-reactive cells required to produce 100,000 splenic PFC in the normal immune response to sheep erythrocytes would be in the vicinity of 1000. This figure, taken together with those obtained with the limiting dilution and double transplantation techniques (Kennedy et al., 1966), immediately casts doubt on the notion that the "background" PFC (which often amount to no more than 10 per spleen) are the cells

which are the precursors of the bulk of PFC appearing in the spleen in response to sheep erythrocytes (Jerne et al., 1963; Hege and Cole, 1966; Aisenberg and Wilkes, 1967).

Additional evidence that "background" PFC are not the sheep erythrocyte antigen-reactive cells, or at least do not make up the sum total of antigen-reactive cells in the spleen, has come from various sources. Miller et al. (1965) and Hege and Cole (1967) showed that neonatal thymectomy did not decrease the "background" PFC number yet the primary immune response of neonatally-thymectomized mice to sheep erythrocytes was severely depressed. Injections of PHA and typhoid-paratyphoid vaccine increased the number of PFC in the spleen yet decreased the immune response to a subsequent injection of sheep erythrocytes (Hege and Cole, 1967). Cheng and Trentin (1967a) injected heat killed enteric bacteria from conventionally-reared mice into specific pathogen-free mice and recorded an increase in the number of splenic anti-sheep erythrocyte PFC several days later. Piglets reared in an environment and fed diets largely devoid of known antigenic material did not have any "background" PFC yet they responded to sheep erythrocytes by producing PFC in the spleen (Sterzl et al., 1965). Germ-free and specific pathogen-free mice raised in some laboratories contained few or no PFC in the spleen (Cheng and Trentin, 1967a) although near normal levels have been reported (Miller, Dukor, Grant, Sinclair and Sacquet, 1967; Nordin, 1968). The environment and the food of mice reared in germ-free isolators could be expected to be free of viable microorganisms but considerable antigenic stimulation may be provided by killed organisms and bacterial products.

The incidence of "background" PFC therefore seems to be related to the immunological history of the animals. It has been suggested that their presence represents the outcome of a degree of cross-reactivity between sheep erythrocytes and various antigenic

determinants encountered in every day life. Anti-sheep erythrocyte "background" PFC differ from "background" anti-horse erythrocyte haemolysin-producing cells and doses of whole body x-irradiation exceeding 200 rads are required to significantly decrease their number either 2 or 7 days later (Hege and Cole, 1967). Interestingly, they are not sensitive to mouse anti-sheep erythrocyte sera of high 19S or 7S antibody titre (Wigzell, 1967; Hege and Cole, 1967) yet the depressive effect of hyperimmune sera on the primary antibody response to sheep erythrocytes has been well documented (Henry and Jerne, 1968; Uhr and Möller, 1968). Szenberg and Cunningham (1968) and Cunningham (1968a) examined "background" PFC in mice and reported that very few were in DNA synthesis, that few were classical plasma cells, and the finding which is particularly pertinent to the discussion in the next subsection, was that the large majority were lymphocytes.

(4) Small lymphocytes as antigen-reactive cells

The property of antibody production is now known not to be invested solely in the plasma cells and their immediate developmental precursors. Mononuclear cells, some of which are indistinguishable from classical small lymphocytes on light microscopic examination, contain (Baney et al., 1962; van Furth, Schmit and Hijmans, 1966b) and secrete antibodies (Attardi, Cohn, Horibata and Lennox, 1964; Harris, Hummeler and Harris, 1966; Hummeler, Harris, Tomassini, Hechtel and Farber, 1966; Cunningham, 1968a). Tubules of endoplasmic reticulum and/or an abundance of ribosomes have, however, been demonstrated in many of these lymphoid antibody producers (Harris et al., 1966; Hummeler et al., 1966). As emphasised by Cunningham (1968), the differences of opinion concerning the relative roles of lymphoid and plasma cells as antibody producers may be more apparent then real. From the proposed genealogy of plasma cells (e.g. Nossal, 1965) there seems

to be no reason why an almost continuous histological picture of antibody-forming cell types, from lymphoid to plasmacytoid cell, should not be encountered. The lack of intracellular organelles characteristic of protein synthesis does suggest, however, that any postulate on the role of the small lymphocyte in antibody production should invoke functions other than secretion of antibody molecules.

The immunological activity of small lymphocytes has been suspected ever since the demonstration that regional lymph nodes were intimately involved with antibody production (McMaster and Hudack, 1935; Ehrich and Harris, 1942). Small lymphocytes predominate in unstimulated nodes and their absolute number is increased in stimulated nodes (Nossal and Mäkelä, 1962; Miller, J.J., 1964). Many early experiments demonstrated that the adoptive transfer of immune reactions could be achieved successfully with lymphocyte-rich inocula (Landsteiner and Chase, 1942; Billingham, Brent, and Medawar, 1956; Harris and Harris (reviewed in Harris and Harris, 1960), Mitchison, 1957; Dixon, Weigle and Roberts, 1957; Stavitsky, 1958; Sterzl, 1959; Makinodan, Gengozian and Shekarchi, 1958). In those systems involving antibody production, most investigators used cells harvested from sensitized donors and the findings were of value in identifying the organ, rather than the cell type, responsible for antibody production. In addition, the relative roles of the host and donor cells were not ascertained and a further limitation was that, in many cases, the host could have mounted an effective host-versus-graft reaction (discussed at length by Mitchison, 1957; Gengozian, Makinodan and Shekarchi, 1961). Largely from the work of Gowans and McGregor and their colleagues, the small lymphocyte has been elevated recently to the status of antigen-reactive and immunologically competent cell. Physiologically, it is admirably suited for candidacy as the precursor of both antibody-producing plasma cells and the sensitized cells believed to be involved in cell-mediated immune responses. The capacity for

extensive migration through lymphoid organs (Section IA) would enable this cell type to be deployed most effectively in sites of antigen accumulation (e.g. Fitzgerald, 1964; Gowans and McGregor, 1965).

Rats, drained of thoracic duct lymphocytes over a period of many days, failed to respond by producing normal amounts of antibody to tetanus toxoid and sheep erythrocytes. This finding provided circumstantial evidence that lymphocytes were involved in primary antibody responses. Cellular inocula, containing more than 99% viable thoracic duct small lymphocytes, restored the response of chronically-drained rats to normal levels (Gowans, McGregor, Cowen and Ford, 1962; McGregor and Gowans, 1963). The immunological deficiency in chronically-drained rats was shown not to be due to the effects of an adrenal-mediated stress reaction or chronic restraint and was also shown not to be absolute. Lymphocyte-depleted rats responded quite well to large doses of sheep erythrocytes and the capacity to reject skin homografts was dependent upon the genetic disparity of the skin graft donor and the chronically-drained host. The rejection time was prolonged only when donor and host differed at weak histocompatibility loci (McGregor and Gowans, 1964; McGregor 1966).

Lymphoid cell preparations had been shown to abrogate an existing state of immunological tolerance (Billingham, Brent and Medawar, 1956) and Gowans et al. (1962) and Gowans, McGregor and Cowen (1963) demonstrated that inocula rich in small lymphocytes were able to effect rejection of tolerated grafts. Host rats were rendered tolerant at birth by injections of appropriate F_1 spleen cells (Gowans et al., 1962) or F_1 bone marrow cells (Gowans et al., 1963). Variations in skin graft survival times were striking and probably reflected the degree of cellular chimaerism in the tolerant hosts (Billingham, 1963; Gowans and McGregor, 1965).

The Gowans group went on to show that small lymphocytes were able to restore the haemolysin response in rats rendered unresponsive to sheep erythrocytes by sublethal (Gowans et al., 1962; McGregor, McCullagh and Gowans, 1967) and lethal (Ellis et al., 1967) doses of whole body irradiation. The response in sublethally-irradiated rats could be increased significantly by yeast extracts which were, in point of fact, as effective as large numbers of spleen cells (McGregor et al., 1967). Host responsiveness may therefore have contributed to the measured haemolysin response in irradiated recipients of small lymphocytes. Crucial to the argument that donor small lymphocytes were providing cells with the potential for initiating antibody production, was the demonstration that small lymphocytes from rats rendered unresponsive to sheep erythrocytes were vastly inferior to cells from normal rats in this system. Even though it is unknown whether tolerance in the donors was specific for sheep erythrocyte antigens only, the use of small lymphocytes from such donors strongly emphasises that the response of the irradiated recipients cannot be elevated simply by injecting large numbers of lymphocytes from any source.

The fate of small lymphocytes has been investigated in recipients in which the inoculated lymphocytes induce a graft-versus-host reaction. Taking advantage of the differential uptake of tritium-labelled RNA and DNA precursors by small and large lymphocytes, Gowans (1962) showed that, in the presence of foreign histocompatibility antigens, small lymphocytes differentiated into large cells which were pyronin positive in histological preparations. Large pyroninophilic cells incorporated tritiated thymidine and the appearance of increasing numbers of lightly-labelled small lymphocytes in subsequent days, suggested that the DNA-synthesizing large pyroninophilic cells divided to produce small lymphocytes (Gowans et al., 1962, Gowans, 1962), some of which may possess the property of migrating from blood to lymph (Ford, Gowans and McCullagh, 1966). A point often stressed (e.g. Gowans

and McGregor, 1965), and which is suggestive of a functional heterogeneity in the thoracic duct small lymphocyte population, is that only a small number of inoculated lymphocytes differentiate to large pyroninophilic cells under the impact of foreign histocompatibility antigens.

The simplest hypothesis relating these histological events to the proposed function of the small lymphocytes as an immunologically competent or antigen-reactive cell, is that small lymphocytes respond to antigen by differentiating and proliferating to produce a progeny of immunocytes (whether they be sensitized lymphocytes or antibody-forming cells) through the intermediary of the large pyroninophilic cell. The clonal expansion of lymphocytes in this manner also provides a mechanism for the development of specific immunological memory. The anamnestic response, as measured by an increased level of circulating antibody or a decreased rejection time of homografts, could be a function of an increased number of reactive or competent cells. The emergence of different classes of antibody or the production of more avid antibody molecules could be a function of either a selective increase in the number of certain types of reactive cell or, alternatively, a qualitative change in the original antigen-reactive cell type. Gowans and Uhr (1968) have transferred secondary responsiveness to ØX174 bacteriophage to sublethally-irradiated recipient rats with purified populations of thoracic duct small lymphocytes collected from donor rats primed 1.5 to 15 months previously. The incubation procedure used to eliminate the majority of large and medium lymphocytes was shown not to involve the differentiation of these cells to small lymphocytes since the proportion of lymphocytes, which had originally incorporated tritiated thymidine, <u>decreased</u> during the course of incubation. "Incubated inocula" were in fact more potent than freshly-collected thoracic duct cells when equal numbers of cells were injected into irradiated recipients together with Ø X174.

The results of the Gowans and Uhr (1966) experiments were clear cut and demonstrated unequivocally that thoracic duct cell inocula, rich in small lymphocytes, are very effective in transferring secondary responsiveness adoptively to syngeneic irradiated recipients. Interpretations, however, are complicated by the fact that sublethally-irradiated recipients were used and the contribution of host cells to the heightened antibody response cannot be discounted. The finding that appropriately-sensitized, chronically-drained rats were still able to respond normally, or in a secondary fashion, to antigens such as sheep erythrocytes, tetanus toxoid, and Ø X174 bacteriophage, suggests that a sessile population of sensitized and reactive cells persist within the tissues of drained rats (McGregor and Gowans, 1963; Gowans and Uhr, 1966). This population may or may not be related to the reactive cells which are removed by chronic thoracic duct drainage. Finally, Ellis et al. (1967) recently demonstrated that thoracic duct lymphocytes from primed donor rats were highly effective in transferring tetanus toxoid responsiveness to lethally-irradiated rats. By using a combination of immunofluorescent and autoradiographic techniques, they showed that the DNA-synthesizing, antibody-forming cells in the spleens of the heavily-irradiated recipients soon after transfer were not derived from medium and large lymphocytes. The antibody formers were therefore most likely to have evolved from nonDNA-synthesizing small lymphocytes in the inoculum. Thus "...the candidature of the small lymphocytes for the precursor cell (of the antibody-producing cell) is very strong but the crucial test has yet to be performed" (Ellis et al., 1967).

(5) Evidence for synergism in haemolysin production

A new lead in the investigations into the underlying cellular mechanism of haemolysin production was provided in 1966 by Claman and his collaborators. Claman, Chaperon and Triplett (1966a) demon-

strated that a mixture of thymus and bone marrow cells, when injected into syngeneic irradiated mice, produced a greater haemolysin response to sheep erythrocytes than could be accounted for by summating the response of mice injected with either thymus or marrow cells alone. Using the assay system of Playfair et al. (1965), they firstly confirmed the finding of Kennedy et al. (1965a) and Playfair et al. (1965) that a linear relationship existed between the number of spleen cells injected and the number of "active areas" of haemolysis appearing in the spleens of the irradiated recipients. In addition to the number of active areas, they also determined the "specific activity" of the spleens, the latter being a measure of the proportion of spleen segments with haemolytic activity. Mice injected with sheep erythrocytes and either 50 million syngeneic thymus cells or 10 million syngeneic bone marrow cells contained no more haemolytic activity in their spleens than control mice injected with the antigen only. Spleens of mice receiving both thymus and bone marrow cells contained many more, and apparently larger, zones of haemolysis. In further experiments, in which either the thymus or bone marrow inoculum was kept constant at 20 million and 10 million cells, respectively, a linear response curve was obtained over a certain range with increasing doses of the other cell type. On the basis of their observations, Claman et al. (1966a) proposed that "auxillary" cells and "effector" cells were involved in the haemolysin response to sheep erythrocytes. The basic findings of the Claman group have been confirmed (Cheng and Trentin, 1967b).

Claman et al. (1966b) extended these experiments and measured serum haemolysins in the irradiated mice as well as "active areas" and "specific activity" in the recipient spleens. In experiments involving groups of only 4 to 6 irradiated mice, no synergism with adult bone marrow was obtained when thymus cells from one day old donors were used in contrast to the situation in which thymus cell donors were 6 days old or adult. Synergism was demonstrated in

recipients of 50 million thymus and 1 million adult spleen cells but not in recipients of 10 million bone marrow and 1 million adult spleen cells. One million spleen cells were as effective as 10 million bone marrow cells when combined with 50 million thymus cells. Thymus cells from irradiated donors were inactive and attempts to substitute subcutaneously placed thymus grafts for thymus cells were also unsuccessful. By delaying the injection of one or other of the cell types, Claman et al. (1966b)hoped to show in which population the "auxillary" cell resided. No difference was apparent, however, in any of the groups in which either bone marrow or thymus cells were initially stimulated with sheep erythrocytes and the other cell type injected two days later.

In a recent report, Claman, Chaperon and Selner (1968) used the Jerne plaque-forming cell assay as well as serum haemolysin determinations in an analysis of the interaction between thymus and bone marrow cells. This study, combined with another from the same group (Chaperon, Selner and Claman, 1968), clearly demonstrated that the number of splenic plaque-forming cells may give a false picture of the haemolysin-producing status of the irradiated animal. The effect of varying the route of injection of one or other of the cell types was determined in experiments again involving only very few mice per group. The serum titre was not affected by injecting either bone marrow or thymus cells intravenously or intraperitoneally. By contrast, significant numbers of PFC were present in the spleen only when bone marrow was injected intravenously. This finding can be interpreted to indicate that the site of residence of haemolysin-forming cells depends on the site of residence of the majority of inoculated bone marrow cells. Bone marrow cells could be expected to home in greater numbers to the spleen when injected intravenously rather than intraperitoneally (e.g. Bainbridge, Brent and Gowland, 1966). Massive doses of sonicated thymus cells or rat thymus cells injected

daily, did not mirror the effects of untreated syngeneic thymus cells when injected together with bone marrow cells and sheep erythrocytes on the day of irradiation. Thymus segments placed intraperitoneally were effective in increasing the haemolysin response in mice injected intravenously with bone marrow cells but irradiated thymus segments were inactive. In addition, the presence of the thymus in the irradiated recipient was shown not to be important in the interaction between inoculated thymus and marrow cells in the production of haemolysins. As many splenic plaque-forming cells were present in adult-thymectomized, irradiated mice as in sham-thymectomized, irradiated mice injected with 50 million thymus cells and 10 million bone marrow cells and the serum haemolysin titre was identical in both groups.

Claman et al. (1968) were unable to determine which population contained the "auxillary" cells and which contained the "effector" cells. In their system, allogeneic combinations of cells failed to interact in the production of haemolysins (Chaperon and Claman, 1967) and measures to identify the immunogenetic character of the antibody-forming cell could not be instituted. Claman et al. (1966a) suggested that the bone marrow provides the "effector" cells and the thymus the "auxillary" cells. Radovich, Hemingsen and Talmage (1968) have presented data which they believe favours the alternative hypothesis. Nonprimed and primed spleen cells, when injected with bone marrow cells into irradiated mice, were effective in promoting the appearance of haemolysin plaque-forming cells in numbers exceeding the summated activities of spleen cells or bone marrow cells alone. Even though standard errors of the mean responses were very large and group sizes small, the finding does contrast with that of Claman et al. (1966) in which evidence for synergism was obtained in mixtures of thymus and spleen and not mixtures of bone marrow and spleen. Radovich et al. considered that the enhancement in the response

was roughly proportional to the extent of haemopoietic regeneration following bone marrow inoculation. This led to the hypothesis that the regenerating spleen provided a more suitable milieu for the trapping and/or differentiation and/or proliferation of antibody-forming cells or their precursors, resident in the spleen cell inoculum or, in the case of the Claman et al. experiments, in the thymus cell inoculum. It is obvious that the controversy will persist until such time as chromosomal, immunogenetic, or immunoglobulin allotype markers are introduced into the system.

Davies, Leuchars, Wallis, Marchant and Elliott (1967) and Davies, Leuchars, Wallis, Sinclair and Elliott (1968b) have presented evidence in favour of the concept of a thymus-marrow interaction in the immune response to sheep erythrocytes. Their system employs thymectomized, irradiated CBA/Cbi mice injected with CBA/Cbi bone marrow cells and grafted with a chromosomally-marked allogeneic thymus graft from CBA/H-T6T6 mice. It is based on a system devised by Doria, Goodman, Gengozian and Congdon (1962) for the analysis of the immunological activity of cells in allogeneic radiation chimaeras. Thirty days after irradiation, bone marrow protection,and thymus grafting the chimaeras were immunized with two injections of sheep erythrocytes and mice with high antibody titres selected as donors of spleen cells. These cells were injected together with sheep erythrocytes into irradiated CBA/Cbi and CBA/H-T6T6 mice, some of which had been presensitized against CBA/H-T6T6 and CBA/Cbi histocompatibility antigens, respectively. High anti-sheep erythrocyte antibody titres were obtained in nonsensitized mice and in isoimmune CBA/Cbi mice which were capable of eliminating thymus graft-derived cells. In secondary recipients sensitized against CBA/Cbi cells (i.e. bone marrow-derived cells), no antibody response was obtained (Davies et al., 1967). Previous work had shown that, in thymectomized

irradiated mice protected with bone marrow cells and grafted with thymus tissue, the majority of cells in mitosis in the spleen and lymph nodes were of bone marrow type. A striking increase in the number of mitotic figures of thymus graft cell type was observed 2 to 3 days after sheep erythrocyte challenge (Leuchars et al., 1964; Davies et al., 1966). This burst of mitosis coincided with a sharp increase in the number of large pyroninophilic cells in the spleen.

The observations in this selective transfer system were extended to include the primary immune response to sheep erythrocytes (Davies et al., 1968b). The haemolysin titrations and Jerne plaque-forming cell assays clearly showed that the presence of cells of bone marrow genotype was absolutely essential to effect a response to sheep erythrocytes in the secondary hosts. No conclusions could be reached on the requirement of thymus graft-derived cells in the primary immune response to sheep erythrocytes in the secondary hosts since the haemolysin titrations and PFC assays gave quite different results. In discussion, Davies et al. (1968b) favoured the notion that thymus-derived cells influence the immunological activity of bone marrow-derived cells by means of a humoral factor especially in the secondary response to sheep erythrocytes.

The only point which may be a serious criticism of the experimental design and which would negate the validity of the conclusions, is that cells were transferred from the primary hosts up to 51 days after thymus grafting. The rapid replacement of graft lymphocytes with exogenous cells has been well documented in several systems (Section IA). It is quite conceivable that at the time of antigen challenge, and certainly by the time of spleen cell transfer, the graft had been completely replaced by bone marrow-derived cells which may have migrated in large numbers from the graft. Such thymus graft migrants would be of CBA/Cbi type and would be rejected in the appropriate secondary hosts together with the bone marrow-derived cells which had not migrated

through the thymus. The possibility cannot be excluded that the bone marrow-derived, antibody-forming cell precursors had been resident at some stage in the thymus graft. The continued production of thymus donor type cells within the thymus graft, and subsequent emigration from the graft, appears to extend beyond the time of thymus grafting and hence the time associated with much tissue disruption and escape of thymus cells. This evidence has been furnished by experiments in which chromosomally-marked thymus grafts were removed after one week. Fewer cells of graft origin were found dividing in the spleen and lymph nodes of the chimaeric mice (Davies et al., 1968a). Nevertheless, the number of bone marrow cells which had migrated through the thymus graft cannot be determined and the objections raised in the discussion above remain valid.

In conclusion, the present day concept of the antigen-reactive cell can be said to have evolved largely from the speculations of Burnet and the experimentation of Gowans and his colleagues. It has been proposed that antigen-reactive cells, which in many systems are probably small lymphocytes, respond to antigenic determinants by producing a progeny of specific antibody-forming cells through processes involving proliferation and differentiation. Only a small proportion of cells present in lymphoid organs, such as the spleen and lymph nodes, responds to antigen and Kennedy and his colleagues have estimated this number to be 1,000 per normal mouse spleen in the sheep erythrocyte system. The figure of 1,000 may, however, be grossly inaccurate and the actual number of cells responsive to a single antigenic determinant remains unknown. If antigen recognition is a cell surface-mediated phenomenon then the specificity of the interaction between antigen and reactive cell could be achieved by the presence of antibody-combining-site-configurations on the surface of antigen-reactive cells. The question remains open as to whether individual cells are genetically restricted in their reactivity or

whether they are invested with the property of multipotentiality prior to contact with antigen. The concensus of opinion seems to favour the former hypothesis and much speculation now centres around the processes whereby the presumptive mechanisms of somatic mutation and genetic recombination generate the diverse patterns of immunological specificity in antigen-reactive cells or their precursors (Jerne, 1967c).

The end cell in the proliferative and differentiative cellular events of antibody production is probably the plasma cell. The abundance of intracytoplasmic structures, indicative of a protein secretory function, set this cell apart as a fully differentiated antibody producer par excellence. It is obvious from the literature that cells of lymphocyte-like morphology may contain and secrete antibodies and it is unlikely that all these cells are destined to mature into plasma cells. Selective processes may occur at this stage to restrict the maturation of antibody-producing cells and these processes may act at the level of individual clones of cells or at the level of the cells within the "expanding" clones. The antigenic reactivity of some small lymphocytes in the circulating pool has been clearly demonstrated but no direct evidence is available to indicate whether some or all of the responding cells eventually elaborate antibody molecules or produce a progeny of antibody-forming cells. Furthermore, the property of specific antigenic responsiveness is unlikely to be restricted to the population of recirculating small lymphocytes.

The 19S haemolysin-forming cell response to sheep erythrocytes has been investigated at the cellular level in species such as the rat and mouse. Studies on the growth kinetics of splenic plaque-forming cells in the exponential phase of the response have indicated that the rapid increase in plaque-forming cell number can be accounted for most satisfactorily by mechanisms of differentiation from non antibody-producing precursor cells and proliferation of plaque-forming cells

themselves. Many plaque-forming cells at this time are in DNA synthesis and cell proliferation is an invariable accompaniment of haemolysin-forming cell production in the spleen. It has yet to be resolved whether some cells commence antibody production early in the response without undergoing DNA synthesis and cell division. The complicity of at least two cell types in haemolysin production has been inferred from the recent studies of thymus-marrow synergism in irradiated animals. The cell type responsible for antibody production in these systems has not been identified unequivocally.

C. The Immunological Activity of Thymus Cells

(1) The immunological effects of thymectomy

Experiments dating from 1961 have clearly demonstrated that the development and maintenance of an intact immune system, in a variety of higher vertebrate species, is dependent on the presence of the thymus. The immunological effects of thymectomy have been reviewed recently by Miller and Osoba (1967) and it is now quite obvious that thymectomized animals may display defects in immune capacity depending on the age at thymectomy, the species under investigation, the antigen chosen, the age at challenge, the various parameters of the elicitation and detection of the particular immune response and the presence or absence of intercurrent infections. In general, the experiments thus far conducted indicate that the effects of thymectomy are more dramatic the earlier in life that thymectomy is performed. Moreover, cell-mediated immune reactions appear to be affected to a greater extent than humoral immune responses. Serum immunoglobulin levels, and the antigenic and functional characteristics of immunoglobulins in neonatally-thymectomized rats and mice, do not deviate significantly from those of the immunoglobulins in non-thymectomized animals (Humphrey, Parrott and East, 1964; Fahey, Barth and Law, 1965) although IgA levels may be reduced (Arnason, de Vaux St. Cyr and Relyveld, 1964; Arnason, de Vaux St. Cyr and Shaffner, 1964).

The features of certain immunological deficiency syndromes in children, and the studies on the bursa of Fabricius in chickens, suggest that the compromised immunological system following thymectomy in mammals, may be due to the presence of other central lymphoid organs, primarily involved with the development of immunoglobulin- and antibody-producing cellular systems (reviewed in Good, 1966; Warner, 1967; Miller and Osoba, 1967; Goldstein and Mackay, 1969). The

search for an organ, with bursa-like functions, in the gut-associated lymphoid tissues has been the subject of intensive investigations conducted by Good and his colleagues. Attention has been focussed on the lymphoid tissues of the gut since epithelial-mesenchymal associations seem to be a feature of the ontogenetic development of primary lymphoid organs. The thymus and bursa arise as epithelial outpouchings from ectoendodermal junctions (reviewed in Miller and Davies, 1964; Osoba, 1968a) and studies in the embryo indicate that, in the chicken and mouse at least, the lymphoid cells are of mesenchymal origin and are sequestered from the circulation (Moore and Owen, 1967).

Convincing evidence has been presented that the Peyer's patches are required for the expression of full antibody producing capacity in the rabbit (Perey, Cooper and Good, 1968). It remains to be determined whether the response to the antigen used (Brucella abortus organisms) is mediated by the Peyer's patches themselves or whether these lymphoid aggregates, by influencing the responsiveness of peripheral lymphoid organs such as the spleen and lymph nodes, behave in an analogous fashion to the thymus and bursa. Recently, Fichtelius (1968) has proposed that the entire epithelial lining of the intestinal tract is the bursal equivalent in mammals.

Notwithstanding the above considerations, simple extirpation of the thymus in the newborn period has been shown to depress the antibody response to certain antigens in several mammalian species. In some antigenic systems the primary response was unaffected whilst the secondary response was markedly depressed (Hess, Cottier and Stoner, 1963; Hess and Stoner, 1966; Basch, 1966). The antigens used, namely MS-2 coliphage and tetanus toxoid, were quite capable of eliciting a response at the time the thymus was removed. By contrast, neonatal mice do not respond to sheep erythrocytes (Hechtel, Dishon and Braun, 1965) and injections of these erythrocytes resulted in a normal

secondary response in neonatally-thymectomized mice which had responded poorly to the primary injection (Sinclair, 1967; Sinclair and Elliott, 1968a). Moreover, defective haemolytic antibody responses may be less apparent the later in life that neonatally-thymectomized mice are challenged with antigen (Rogister, 1965; Dukor, Dietrich and Rosenthal, 1966; Sinclair and Millican, 1967; Zinzar and Svet-Moldavsky, 1967; Takeya and Nomoto, 1967b). Takeya and Nomoto (1967a) have shown that multiple injections of sheep red cells elevate the antibody response in neonatally-thymectomized mice to levels which ultimately approach those attained in nonthymectomized litter mates injected with antigen for the first time. This finding has been used as evidence that the few competent cells in neonatally-thymectomized mice (vide infra) may be increased in number by exposure to introduced antigens and hence to cross-reacting antigens in the environment.

In a variety of experimental situations, cells from the spleen, lymph nodes, and thoracic duct of neonatally-thymectomized rats and mice have not performed as well, when assayed on a cell for cell basis, as lymphoid cells from intact animals. Thus, Cross,Leuchars and Miller (1964) and Miller, Leuchars, Cross and Dukor (1964) failed to restore the haemagglutinin response to sheep erythrocytes, and the potential for allogeneic skin graft rejection, in adult-thymectomized, irradiated mice with spleen cells from neonatally-thymectomized mice. Spleen cells from normal mice improved the immunological status of such animals (Cross et al., 1964; Miller et al., 1964; Duplan, 1965). Dalmasso, Martinez, Sjodin and Good (1963) showed that 5 million spleen cells from normal two months old mice were fully able to produce runt disease in newborn allogeneic recipients. The spleen cell inoculum, when taken from neonatally-thymectomized mice of the same age, had to be increased to 20 million cells to produce a similar effect. Spleen cells from neonatally-thymectomized mice were required

in larger numbers than normal spleen cells in order to prevent wasting disease and to improve the capacity for tumour graft rejection in neonatally-thymectomized mice (East and Parrott, 1964). Agnew (1967) reported a reduced incidence, and an increased mean latent period, of tumour growth in neonatally-thymectomized rats injected with 10 to 20 million thoracic duct cells from normal syngeneic donors. By contrast, 40 million thoracic duct cells from neonatally-thymectomized rats were unable to modify the susceptibility of other neonatally-thymectomized rats to tumour development. As mentioned in Section IA, thoracic duct cells from neonatally-thymectomized rats were at least 10 times less effective in inducing runt disease in newborn allogeneic recipients than thoracic duct cells from intact rats (Rieke, 1966).

(2) The reconstitutive capacity of thymus cells

Reconstitution experiments in thymectomized animals have provided a means of assessing the immunocompetence of various cellular inocula. Many groups of workers have demonstrated the effectiveness of normal spleen and lymph node cells in preventing wasting disease and improving the immunological status of thymectomized mice and rats (Dalmasso et al., 1963; Miller, 1964a, b; East and Parrott, 1964; Trainin, Law and Levey, 1965; Isaković, Waksman and Wennersten, 1965; Goedbloed and Vos, 1965). The lymphoid inocula, in the case of neonatally-thymectomized recipients, were usually given within two weeks of birth and the immunological performance of the animals assessed some weeks later. In the study of Isaković et al. (1965), however, the reconstitutive capacity of 1×10^9 lymph node cells was demonstrated when inoculated at the time of sensitization with antigen; in this case bovine serum albumin in neonatally-thymectomized rats. In the other systems, the para-

meters used to determine the degree of reconstitution were the haemagglutinin response to sheep red cells, the prevention of wasting disease, the rejection of skin or tumour homografts, and the capacity of recipient lymphoid cells to elicit graft-versus-host (GVH) reactions.

Parallel studies using injections of dissociated thymus cells have usually shown that cells from this source are deficient in their capacity to correct the defects in neonatally-thymectomized mice and rats. 200 million syngeneic adult spleen cells reversed wasting disease in 90% of neonatally-thymectomized mice whereas the same number of thymus cells resulted in recovery of only 30% of the recipients (Hilgard, Yunis, Sjodin, Martinez and Good, 1964). Earlier work by the same group indicated that syngeneic thymus cells increased survival rates in neonatally-thymectomized mice without increasing the immunocompetence of spleen cells, as determined by the splenic GVH assay in appropriate F_1 hybrid recipients (Dalmasso et al., 1963). Large numbers of intraperitoneally-injected syngeneic thymus cells from newborn or adult mice were later shown to improve substantially the immunological performance of spleen cells from neonatally-thymectomized mice. Three hundred to 400 million C3H thymus cells did not result in a GVH reaction in neonatally-thymectomized A strain mice yet spleen cells from these hosts at 2 months of age caused splenomegaly in 8 day old (C3H x DBA)F_1 and (C3H x C57BL)F_1 hybrids; hosts which are presumed to be capable of rejecting A strain cells (Yunis, Hilgard, Sjodin, Martinez and Good, 1964; Yunis, Hilgard, Martinez and Good, 1965). When (A x C3H)F_1 hybrid thymus cells were used in the reconstitution of A strain neonatally-thymectomized mice, spleen cells from some of the animals possessed host type reactivity but donor type reactivity could be demonstrated more readily (Yunis, Martinez, Smith and Good, 1964).

The discriminating assay of cell chimaerism described by Simonsen (1962) was used in all the allogeneic and semi-allogeneic reconstitution experiments. The inability of huge numbers of C3H thymus cells, or even 100 million spleen cells (Yunis et al., 1965), to induce a GVH reaction in the thymectomized A strain recipients remains unexplained. The data from the GVH assays, coupled with the skin graft data (Yunis et al., 1965), does indicate, however, that the reconstitution of neonatally-thymectomized mice with thymus cells is dependent upon the immunological activity of the inoculated cells.

East and Parrott (1964) and Law (1966a) were able to prevent wasting and improve the immunological status of neonatally-thymectomized mice with multiple injections of thymus cells. Miller (1964a, b) and Trainin et al. (1965) reported that relatively small numbers of syngeneic spleen and lymph node cells, but not thymus cells, reconstituted neonatally-thymectomized mice. Trainin et al. (1965) and Law (1966a) observed that many thymectomized recipients, injected with single cell suspensions of lymph node or spleen on the second day of life, died between 3 and 6.5 months of age. The protection afforded by these inocula was therefore temporary and may reflect a limited life span of the component cells or their progeny within the tissues of the recipients. Goedbloed and Vos (1965) reported that lymph node cells were 10 to 20 x as effective as thymus cells in restoring the capacity for skin graft rejection in adult-thymectomized, irradiated mice protected with small numbers of bone marrow cells.

Thymus cells injected soon after, or some weeks prior to a footpad injection of BSA in Freund's adjuvant, did not improve the defective response of neonatally-thymectomized rats with respect to delayed or Arthus type skin reactivity or anti-BSA antibody production (Isaković et al., 1965). Best reconstitution could be achieved with lymph node cells given at the time of sensitization and a delay of 2 to 4 days reduced the efficacy of such inocula. Unfortunately, these latter conditions

were employed in testing for the reconstitutive capacity of thymus cells in this system. The response to the footpad deposit of antigen was presumably mediated by cells in that site and the regional lymph node. No increase in organ size, comparable to that following lymph node cell injection, occurred in the lymph nodes of thymus cell-inoculated rats. It is possible that the thymus cells did not home in large numbers to the lymph node (Section IA) where contact with antigen was presumably occurring. Interestingly, the weight and cellularity of the spleen increased with injections of either lymph node or thymus cells. Perhaps different results would have been obtained with the use of another systemically administered antigen, the response to which is mediated in the spleen by resident or migratory cells.

As mentioned previously, the degree of reconstitution of neonatally thymectomized mice using spleen, lymph node, or thymus cells was determined several weeks after injecting the cells in the neonatal period. One exception to this general pattern exists in the literature and the results seem to be at variance with the conclusion that thymus cells are not as effective as lymph node cells in reconstitution experiments. Taylor (1963) injected 50 million adult syngeneic lymph node or thymus cells into groups of 7 weeks old neonatally-thymectomized CBA mice and challenged them with BSA in Freund's adjuvant 7 weeks later. The antibody titres, as measured by the antigen-binding capacity of the serum, were increased over those in uninjected controls by a factor of 10 and to an equal extent in both groups. Furthermore, by using chromosomally-marked thymus and lymph node cells, both cell types were later detected in the spleens and lymph nodes of the hosts. It seems, therefore, that the later in life that thymus cells are injected, the greater is the probability of successfully reconstituting neonatally-thymectomized mice. Perhaps the demand for cells intensifies with increasing age and it is tempting to postulate that antigenic stimulation

increases the colonization potential of thymus cells by means of clonal expansion. In the relatively protected environment of the neonatal mouse in which maternal antibodies are present, thymus cells, unlike lymph node and spleen cells, may have a limited life span. The adult thymus may also be protected from most antigenic insults and, as discussed in Section IA, several pieces of evidence suggest that many thymus lymphocytes die within the organ.

(3) Activity of the thymus in adoptive transfer and in vitro systems

The use of heavily-irradiated mice as recipients of thymus cells has established unequivocally that the thymus does not contain haemopoietic stem cells. Thymus and thoracic duct lymphocytes, when injected into lethally-irradiated isologous recipients, are unable to effect lymphoid regeneration of the thymus or erythromyeloid regeneration of the spleen and bone marrow (Gesner and Gowans, 1962b; Ford and Micklem, 1963; Micklem, Ford, Evans and Gray, 1966; Ford, 1966a). By contrast, the bone marrow contains cells capable of permanently repopulating the entire lympho-myeloid complex in irradiated mice (e.g. Micklem et al., 1966) and haemopoietic stem cells are present in peripheral blood (Popp, 1960; Goodman and Hodgson, 1962; Barnes and Loutit, 1967).

Fragments of thymus have been shown to potentiate antibody production in cultures of spleen from immunized mice (Saunders and King, 1966) or lymph node fragments from immunized rabbits (Wolf, 1968). Metcalf (1968) observed a slight potentiation of in vitro bone marrow colony growth with thymus cells in the presence of stimulating serum. These various in vitro systems raise the question as to whether the thymus lymphocytes, the thymus cytoreticulum, or products from either or both of these components, are responsible for the synergistic effect of thymus in cultures of spleen, lymph node or bone marrow. Consideration must also be given to the proposals that

lymphocytes may have non-immunological duties in the regulation of tissue growth and size (e.g. Humble, Jayne and Pulvertaft, 1956; Trowell, 1958; Pulvertaft, 1959; Loutit, 1962; Burch and Burwell, 1965).

In many experiments, the antibody producing activity of thymus cells has been unimpressive, but these cells have certainly not been without effect. Early reports indicated that thymus fragments and single cell suspensions of thymus prepared from immunized donors were able to give rise to antibodies in irradiated recipients or in vitro. The quantity of antibody produced was invariably less than that following implantation, injection, or in vitro culture of lymph node, spleen or peritoneal exudate cells (Fagraeus, 1948; Thorbecke and Keuning, 1953; Stoner and Hale, 1955; Askonas and White, 1956; Dixon et al., 1957; Stoner and Bond, 1963; Thorbecke and Cohen, 1964). Antibody titres in extracts of thymus from immunized animals were found to be very low and the quantity of antibody could be accounted for by the volume of serum calculated to be contained within the thymus (Bjørnboe, Gormsen and Lindquist, 1947; Harris, Rhoads and Stokes, 1948).

In the in vitro systems of Askonas and White (1956) and Thorbecke and Cohen (1964), bone marrow cells were surprisingly potent in elaborating both antibodies and gammaglobulins. Gengozian, Makinodan and Shekarchi (1961) clearly demonstrated that bone marrow cells from sensitized mice were quite capable of transferring rat erythrocyte reactivity to syngeneic irradiated recipients. This was especially so in the case of donors immunized several weeks previously. Van Furth, Schuit and Hijman (1966a) ascribed the high immunoglobulin producing activity of human bone marrow preparations in vitro to a few resident plasma cells. Since no immunoglobulin-positive cells, of the type presumed to be the developmental precursors of plasma cells, were present in the bone marrow, these workers concluded that the mature

plasma cells had migrated from other sites. Friedman (1964) injected several strains of mice with 2×10^9 sheep erythrocytes and found fewer plaque-forming cells (Section IB) per million inoculated cells in the thymus and marrow than in non-lymphoid organs such as the lung and liver.

Chaperon et al. (1968) have recently extended the observations of Gengozian et al. (1961) and Friedman (1964). Both 19S and 7S plaque-forming cells (PFC) were enumerated in the spleen, thymus and bone marrow after an injection of sheep erythrocytes. The number of 7S PFC in the bone marrow and thymus increased progressively over the 42 day observation period. At later time points the number of 7S PFC per 10^7 cells in the bone marrow actually exceeded that in the spleen. The activity of cells from the three organs, in transferring sheep erythrocyte reactivity adoptively to irradiated recipients, closely paralleled the appearance of 7S PFC in the organs. In the transfer experiments the bone marrow was approximately 10x as effective as thymus cells and at all times contained more 19S and 7S PFC per 10^7 nucleated cells. These authors considered that both 7S PFC and antigen-reactive cells (Section IB) differ from 19S PFC in that they migrate extensively between organs after primary immunization. However, the presence of 19S PFC in the blood has been well documented (Kearney and Halliday, 1965; Friedman, 1964; van Furth et al., 1966b; Hiramoto, Hamlin and Harris, 1968) but their life span in the blood and tissues, at least as 19S haemolysin-forming cells, seems to be limited.

Thymus cells from normal mice were essentially unable to adoptively transfer sheep or rat erythrocyte haemolysin and haemagglutinin responsiveness to irradiated or cyclophosphamide-treated mice (Thorbecke and Cohen, 1964; Kennedy et al., 1965a; Santos and Owen, 1966; Claman et al., 1966a, b; Papermaster, 1967). Furthermore, normal rabbit thymus cells differed from lymph node cells in that

they were ineffective in adoptively transferring, to homologous irradiated recipients, antibody responsiveness to Shigella paradysenteriae (Harris and Harris, 1955). Anti-BSA antibodies were detected in diffusion chambers containing suspensions of homologous thymus cells and BSA. Significant amounts to antibody were present 7 to 10 days after intraperitoneal implantation of the chambers into newborn rabbits (Holub, Říha and Kamarýtová, 1965). Several criticisms of these experiments may be raised. The responsiveness of newborn rabbits with diffusion chambers containing killed or irradiated thymus cells, and injected intraperitoneally with BSA, does not seem to have been assessed. The thymus cells may have produced antibody or they may have absorbed antibody which had been elaborated elsewhere. Thymus cell suspensions from hyperimmunized rabbits absorb I^{131} BSA in vitro and this may in turn reflect the presence of passively absorbed anti-BSA antibody (Horvàth, 1963). Moreover, in the data presented by Holub et al. (1965), no clear cut dose response with various cell numbers was apparent and substantial amounts of antibody were present in chambers containing alveolar macrophages.

Background rosette-forming cells, of the type described by the Biozzi group (e.g. Biozzi, Stiffel and Mouton, 1966) and Zaalberg (1964), are readily detected in various tissues of unimmunized mice. The lowest number of such cells, using sheep and chicken erythrocytes, was found in thymus cell suspensions (Zaalberg, 1964; Laskov, 1968). Furthermore, Dent and Good (1965) were unable to detect many rosette-forming cells or plaque-forming cells in the thymus and bursa of chickens immunized with sheep red cells. Landy, Sanderson, Bernstein and Lerner (1965) found that antibody-forming cells were present in the thymus of rabbits 5 days after injection of Salmonella enteritides somatic antigens. The appearance of these cells in the thymus coincided with a striking disruption of the normal thymus architecture. Germinal centres, lymphoid follicles and plasma cells are rarely found in the intact thymus subsequent to parenteral injections

of antigenic material (e.g. Thorbecke and Keuning, 1953; Askonas and White, 1956). Injections of antigens directly into the thymus, infliction of mechanical damage to the thymus, or subjecting the animals to chronic stressing procedures, does result in the appearance of intrathymic plasma cells and/or germinal centres; entities which are considered to be characteristic of antibody-producing tissues (Marshall and White, 1961; Sherman, Adner and Dameshek, 1964; Blau and Waksman, 1964; Sainte-Marie 1965; Hirata-Hibi, 1967). Architectural disruption may have contributed to the observation that plasma cells and germinal centres appear in subcutaneous autografts of rat thymus after parenteral injections of diphtheria toxoid (Stutman and Zingale, 1964). It is not known whether plasma cells and follicular cells are derived from resident thymocytes or whether they are extrathymic in origin. They may have infiltrated via the route of injection or during the course of the inflammatory response subsequent to disruption of the thymus parenchyma.

The results of immunofluorescence studies on the location of immunoglobulin-producing cells in the human thymus strongly suggest that these cells may not be derived from thymocytes but from circulating cells. IgG-and IgA-containing cells were concentrated in perivascular sites mainly in the interstitial connective tissue (van Furth et al., 1966a). Siegler (1965), in a histological study of follicle and germinal centre formation in the thymus of NZB (Burnet and Holmes, 1962, 1964) and aged Swiss mice, concluded that these structures arose in the perivascular connective tissue as they did in many other non-lymphoid organs.

As in the studies of Siegler (1967) and van Furth et al. (1966a), the distribution of plasma cells in the rat thymus led Sainte-Marie (1965) to the conclusion that intrathymic plasma cells were not derived from precursors within the thymus. Mature plasma cells, but virtually no plasmablasts, were found scattered, rather than clustered, in peri-

vascular sites within the trabeculae and less commonly in the parenchyma. These sites coincided with the thymic distribution of intramediastinally injected antigenic material (Sainte-Marie, 1963). All such histological evidence certainly cannot be considered definitive and the conversion of lymphocytes, produced within the thymus, to plasma cells, other antibody-producing cell types, or follicular and germinal centre cells, must remain an open question. Similar considerations apply to the transformation of bone marrow cells into antibody-producing cells but there is clear evidence from the literature that the bone marrow is more active in the production of immunoglobulins than the thymus. It is not possible to decide whether bone marrow cells per se are more intimately concerned with this specialized function or whether the bone marrow differs from thymus in containing a higher proportion of "contaminant" immunoglobulin-producing cells.

(4) The activity of thymus cells in GVH reactions

By definition (Medawar, 1963), the GVH reactivity of single cell suspensions may be considered a measure of the immunocompetence of the entire cell population. Differences observed with various inocula may be a function of qualitative and/or quantitative differences in the cellular components of the inocula. The pathogenesis of GVH reactions is undoubtedly complex (Simonsen, 1962; McBride, 1966; Billingham, 1967) and many findings seem to cast doubt on whether immunological phenomena, as currently visualised, are solely responsible for the observed reaction (reviewed in Miller, 1968). The activity of thymus cells in initiating GVH reactions has been the subject of numerous investigations and the findings will be examined in detail in succeeding paragraphs. As a general conclusion it can be said that mouse and, more particularly, rat thymus cells perform erratically and are inferior to spleen and lymph node cells.

In localized GVH reactions, such as the normal lymphocyte transfer reaction, rat and guinea pig thymocytes were far less effective in producing local lesions than lymph node, spleen or thoracic duct cells (Brent and Medawar, 1966; Ford, 1967; Wilson and Billingham, 1967). Using allogeneic strain combinations, Billingham and co-workers found that thymus cells from rats and mice were inferior to spleen, lymph node, peripheral blood and thoracic duct cells in inducing runt disease and conferring homograft tolerance in newborn animals (Billingham and Brent, 1959; Billingham and Silvers, 1961, 1964; Billingham, Defendi, Silvers and Steinmuller, 1962). The reduced efficacy of thymus cells was particularly striking in the induction of runt disease in Lewis-BN strain rat combinations (Billingham et al., 1962). Goldschneider and McGregor (1966) injected newborn rats with large numbers of allogeneic bone marrow and thymus cells and found evidence of a GVH reaction in very few of the recipients. The minimal activity of bone marrow cells could be further reduced, and in fact abolished with the cell doses used, by taking bone marrow cells from rats chronically drained of circulating lymphocytes. The weak but discernible activity of normal bone marrow cells was thus ascribed to itinerant circulating immunocompetent small lymphocytes (McGregor, 1968). The deleterious effects of restraint on the cellularity and size of the thymus presumably precluded any attempts to use thymus cells from chronically-drained rats in similar experiments.

Newborn thymus cells have been shown to be as effective as adult thymus cells in inducing GVH reactions in allogeneic (Cohen Thorbecke, Hochwald and Jacobson, 1963) and parent to F_1 hybrid (Sosin, Hilgard and Martinez, 1966) mouse combinations. Adult spleen cells (unlike newborn spleen cells) were 10 times as effective as thymus cells in producing splenomegaly (Sosin et al., 1966). The

presence or absence of the F_1 host thymus did not influence the GVH activity of the inoculated thymus cells (Sosin et al., 1966) and, in some instances, thymectomy increased the severity of the reaction (Yunis, Martinez and Good, 1967). Even with parental thymus grafts in neonatally-thymectomized F_1 hybrid mice, some evidence for a GVH reaction in the grafted host has been recorded (Stutman, Yunis, Teague and Good, 1968). Tyan, Cole and Nowell (1966) obtained similar evidence for a GVH reaction in appropriate secondary F_1 hosts injected with spleen and lymph node cells from sublethally-irradiated F_1 mice grafted with parental thymus grafts. The conclusion seems warranted that the GVH reaction in these systems is mediated by thymus graft-derived cells. Parental thymus cells from 10 day old mice killed many sublethally-irradiated F_1 hybrid recipients (Kaplan and Rosston, 1959) but preimmunization was necessary before GVH activity could be demonstrated with thymus cells in the parent-F_1 hybrid mouse system of Fiore-Donati, Chieco-Bianchi, de Benedictis and Tridente (1964). Sensitization presumably occurs in peripheral lymphoid organs and the findings of Fiore-Donati et al. (1964) might suggest that sensitized cells infiltrate the thymus. Tyan and Cole (1964) injected 2.5 million adult parental thymus, lymph node, or spleen cells into sublethally-irradiated F_1 mice. All recipients of spleen and lymph node cells died, whereas deaths were recorded in only 1/3rd of the thymus cell recipients.

Experiments designed to test the competence of chicken thymus cells have been conducted by Warner (1964, 1965) and Cain, Cooper and Good (1968). Warner (1964) used the chorioallantoic membrane (CAM) of the chicken embryo as a means of determining the pock-producing (or pock-initiating) capacity of chicken thymus cells. He demonstrated that the number of immunocompetent cells per inoculum was inversely related to the size of the thymus cortex. A reduction in the number of

cortical lymphocytes was achieved by systemic administrations of corticoster oids or by taking thymuses from older birds. Warner concluded that the thymus medulla contained the CAM pock-producing cells. In this system, the bursa of Fabricius was found to contain very few competent cells and both thymus and bursa contained far fewer competent cells per million nucleated cells than the spleen (Warner, 1965). Cain et al. (1968) reported that thymus cells from 10 weeks old chickens were as effective as spleen cells in inducing splenomegaly in allogeneic 14 day old embryos. Bursal cells, on the other hand, were totally inactive. The conclusion that thymus cells are as effective as spleen cells in this system cannot be justified until data is presented on the minimum number of cells required to induce splenomegaly.

Phillips and Thorbecke (1966) injected mice with rat foetal liver cells or spleen, thymus, and bone marrow cells from adult rats. Four times as many thymus cells as spleen cells were required to give the same index of GVH activity. Large numbers of foetal liver or bone marrow cells were without effect.

It is well known that heavily-irradiated mice may be protected from death by injections of allogeneic or xenogeneic bone marrow cells. Isologous and homologous lymphoid cells have been tested for their capacity to inhibit successful bone marrow homo- or hetero-grafting. Lymph node cells were found to be 2 to 30 times more effective than thymus cells in preventing the therapeutic action of foreign bone marrow cells (Vos, de Vries, Collenteur, and van Bekkum, 1959; Congdon and Duda, 1961).

A feature of the initial stages of localized and systemic GVH reactions in vivo is the transformation of a certain number of inoculated small lymphocytes into large pyroninophilic cells which subsequently divide (Gowans, 1962). The transformation of thymus cells in vitro, under the impact of a variety of stimulants, will

therefore be considered before conclusions are made on the GVH reactivity of thymus cells.

(5) The blastogenic response of thymus cells in vitro

The mitogenic and blastogenic properties of phytohaemagglutinin (PHA), first described by Hungerford, Donnelly, Nowell and Beck(1959) and Nowell (1960), are now clearly recognized even though the mechanism of PHA stimulation in vitro remains an enigma (reviewed in Wilson and Billingham, 1967, and Naspitz and Richter, 1968). Lymphoid cells from the tissues and blood of a variety of mammalian species and chickens may show metabolic and morphological changes when cultured in vitro with PHA. The metabolic changes include increased protein, RNA and DNA synthesis. In addition, it has been well documented that small lymphocytes in PHA cultures transform into large pyroninophilic cells which subsequently divide. Whether the end result of this mitotic activity is a progeny of small lymphocytes has yet to be established. The effects of PHA and other non-specific and specific mitogens on small lymphocytes in vitro has added weight to the contention, derived from studies on the immunological activity of small lymphocytes, that these cells are not inert end cells which are incapable of differentiating into any other morphological cell type.

It is apparent from the literature that experiments addressed the question of PHA responsiveness of thymus cells have yielded conflicting results. Thymus cells from young rats (Schwarz and Rieke, 1966; Metcalf and Osmond, 1966) and pigs (Weber, 1966a) undergo blastic changes in response to PHA in vitro. By contrast, in other systems, including the mixed "leucocyte" reaction, blastoid transformation in cultures of thymus cells from rabbits and guinea pigs was minimal (Chapman and Dutton, 1965; Schrek and Batra, 1966;

Oppenheim, Wolstencroft and Gell, 1967). Nevertheless, a vigorous blastogenic response was obtained with a mixture of thymus cells from genetically dissimilar rats (Schwarz, 1966). Successful PHA cultures of foetal and infant human thymus cells have been reported (extensive literature cited by Naspitz and Richter, 1968), but Claman (1966), Claman and Brunstetter (1968), Winkelstein and Craddock (1967) and Knight, Bradley, Oppenheim and Ling (1968) have called attention to the fact that tritiated thymidine incorporation into thymus cells in vitro is initially high and that a second peak of incorporated radioactivity is evident in late cultures. Only the second peak of activity is dependent upon the presence of PHA. From the results of comparative studies with human spleen, lymph node, peripheral blood and thymus cells, the PHA responsive cells in the adult human thymus are considered to be members of a relatively minor population of cells (Winkelstein and Craddock, 1967; Knight et al., 1968). The work of Weber (1966b) suggests that this particular population of cells may be resident in the thymus medulla. Schwarz (1968) has recently calculated that too few peripheral blood lymphocytes were contained in the vasculature of the thymus to account for the observed lymphocyte transformation in rat thymus cell cultures. Nevertheless, the contribution of immigrant circulating cells in perivascular sites or in the thymus medulla cannot be discounted.

Unlike bursal cells, chicken thymus cells underwent morphological alterations in the presence of PHA (Weber, 1967). Moreover, peripheral blood lymphocytes from bursectomized chickens responded normally to PHA whereas blood cells from thymectomized chickens were essentially unresponsive (Greaves, Roitt and Rose, 1968). There is one technical feature of the Greaves et al. experiments which may alter any conclusion arising from the findings. Simple differential sedimentation techniques were used to harvest peripheral blood "lymphocytes" and it is quite conceivable that culture of cells from

lymphopenic thymectomized chickens (Warner and Szenberg, 1962; Cooper, Peterson, South and Good, 1966) contained more nucleated thrombocytes than cultures containing equal numbers of "lymphoid" cells from bursectomized chickens.

The lymph nodes of neonatally-thymectomized mice and adult-thymectomized, irradiated mice contain fewer cells responsive to PHA in vivo. After footpad inoculation of PHA into normal mice, large numbers of pyroninophilic cells appeared in the diffuse cortex of the regional lymph node. This change preceded a considerable germinal centre and plasma cell reaction in the popliteal node. The number of cells in the diffuse cortex following thymectomy is reduced (Section IA) and very few cells in this site responded to PHA by undergoing blastoid transformation (Dukor and Dietric h, 1967). Rieke (1966) and Meuwissen, Bach, VanAlten and Good (1967) reported that transformation rates in PHA cultures of thoracic duct cells and peripheral blood leucocytes from neonatally-thymectomized rats were lower than in control cultures. Davies et al. (1968a) have recently shown that thymus graft-derived cells make up the bulk of mitotic figures in PHA cultures of peripheral blood lymphocytes obtained from adult-thymectomized, irradiated, bone marrow-protected mice grafted with a chromosomally-marked thymus.

In an abstract, Meuwissen et al. (1967) reported that lymphocytes from patients with thymic alymphoplasia failed to transform in PHA cultures. Variable results have been obtained using lymphocytes from agammaglobulinaemic patients (e. g. Elves, Roath and Israels, 1964, c.f. Fudenberg and Hirschhorn, 1964) and it is not know whether these variations can be ascribed to varying degrees of thymic alymphoplasia or malfunction which may or may not be associated with agammaglobulinaemia. Functional derangements in either or both of the thymus-dependent and immunoglobulin-producing systems have been described in children with rare immunological deficiency diseases such as Bruton's agammaglobulinaemia, Di George's syndrome and thymic alymphoplasia

associated with agammaglobulinaemia (discussed by Good, 1966; Miller and Osoba, 1967; Goldstein and Mackay, 1969). The patient of Lischner, Punnett and Di George (1967), with thymic agenesis and normal immunoglobulin levels, is of interest in this regard. Lymphocytes, none of which were likely to have been derived from the patient's thymus and which were not maternally derived, were numerous in the peripheral blood and lymph nodes. In short term cultures using known mitogens such as PHA, antilymphocyte serum, homologous lymphoid cells and also several antigens, the percent blast transformation of the lymphocytes obtained from this patient was negligible.

As a general conclusion, it can be said that the results of PHA stimulation of thymus cells in vitro parallel those obtained with thymus cell suspensions in GVH reactions. Thymus cells behave somewhat fitfully and the question immediately arises as to whether their discernible activity can be ascribed to itinerant circulating lymphocytes in the interlobular and perivascular connective tissues or in the medulla. Alternatively, is the activity due to a low level of immunocompetence on the part of the large number of cortical lymphocytes produced within the thymus ? Contributing factors may include cellular immaturity, qualitative cellular defects, or a relative decrease in the number of competent cells. The inadvertent inclusion of parathymic lymph node lymphocytes into thymus cell suspensions must also be considered. Lymph nodes of considerable size have been described within, or closely adherent to, the thymus capsule in mice and rats (Blau and Gaugas, 1968). Aside from these considerations of thymus cell activity, an impressive and increasing bulk of evidence now exists to suggest that the presence of the thymus is required for maximal PHA stimulation of peripheral blood, spleen, lymph node and thoracic duct lymphocytes. The possibility must be seriously entertained that many peripheral PHA-

responsive cells are emigrant thymus lymphocytes or their descendants. In view of the discussion to follow in the next subsection, the operation of thymic humoral factors may also contribute to the responsiveness of peripheral lymphocytes to mitogenic agents in vivo and in vitro.

Lymph node and spleen cells participate in both antibody production and the various cell-mediated immune responses. Thymus and bone marrow cells engage in both these immunological processes to a limited extent. Viewed in their entirety, the results of the experiments using thymus and bone marrow cells indicate that these two cell types have differential potencies when assayed on the basis of equal numbers of nucleated cells. Bone marrow cells are far more active in immunoglobulin production and thymus cells are far more active in responses involving foreign histocompatibility antigens. As mentioned previously, this observation may not necessarily reflect differences in the potentiality of the lymphoid cells from these two sources. The bone marrow may contain a greater number of plasma cells and their precursors, the thymus may contain more immunologically competent cells. Both functional cell types may be sequestered from the circulation.

(6) Thymus humoral factors

The influence of the thymus on lymphopoiesis and the induction of immunocompetence has been investigated in organ cultures by Globerson (1966) and Globerson and Auerbach (1967). Thymus explants from normal mice induced GVH reactivity in spleen fragments from sublethally-irradiated mice when cultured together in vitro. The efficacy of thymus across a millipore filter strongly suggested that a thymic subcellular humoral factor was responsible for the observed reactivation of the residual cells in the irradiated spleen. Using various parent-F_1 hybrid combinations of spleen from lethally-irradiated mice and normal bone marrow and thymus, Globerson and

Auerbach (1967) further showed that the bone marrow contributed the reactive cells. The GVH activity of thymus cells themselves cannot be discounted from the data but the results with parental irradiated spleen - F_1 hybrid thymus - parental marrow combinations can be interpreted readily in terms of a competence-inducing humoral factor elaborated by the thymus explant.

Several early reports hinted at the possibility that the thymus may influence peripheral lymphopoiesis via a humoral mechanism (e.g. Metcalf, 1956; Grégoire and Duchâteau, 1956). The first convincing evidence that the thymus may elaborate a factor(s) with competence-inducing properties, came from investigations into the reconstitutive capacity of thymus tissue enclosed in cell-impermeable millipore diffusion chambers and implanted into neonatally-thymectomized mice. A number of mice implanted at 1 to 2 weeks of age with neonatal or embryonic thymus tissue in diffusion chambers (0.1μ pore size), were able to reject homografts of skin and produce haemagglutinins in response to sheep erythrocytes. Control groups included thymectomized litter mates implanted with empty diffusion chambers. No evidence of lymphoid regeneration was apparent in the tissues of the immunologically-reconstituted mice (Osoba and Miller, 1963; Osoba and Miller, 1964; Osoba, 1965a). Similar experiments performed by another group indicated that, in neonatally-thymectomized mice bearing neonatal thymus tissue in diffusion chambers with membranes of 0.45μ pore size, the incidence of wasting diseases was decreased (Levey, Trainin and Law, 1963), the susceptibility to lymphocytic choriomeningitis virus increased (Levey, Trainin, Law, Black and Rowe, 1963), and sheep red cell haemolysin production increased, albeit slightly in less than 2/3rds of the mice (Law, Trainin , Levey, and Barth, 1964). In this case, no depletion of lymphocytes in lymphoid tissues or peripheral blood was reported. Nevertheless, by calculating from the data, the absolute number of mononuclear cells in the blood, it is evident that a severe deficiency persisted in many of the mice (Levey et al., 1963).

Schaller and Stevenson (1967) were able to reverse the signs of the post-thymectomy wasting syndrome in 30% of mice with 5 syngeneic or allogeneic neonatal thymuses in diffusion chambers constructed with membranes of 0.3μ pore size. Lymphoid hypoplasia persisted in surviving mice and foreign skin grafts were rejected in normal fashion. In keeping with the findings of Osoba (1965a), those mice reconstituted with allogeneic thymuses in diffusion chambers were able to reject thymus donor type skin. Host type GVH activity of spleen cells was demonstrated on transfer to appropriate F1 hybrid newborns.

Thymus tissue enclosed in millipore diffusion chambers has also improved, to some extent, the immunological performance of adult-thymectomized, irradiated mice (Miller et al., 1964; Barclay, 1964; Osoba, 1968b) and neonatally-thymectomized hamsters (Wong, Taub, Sherman and Dameshek, 1966; Sherman, 1967), rats (Aisenberg and Wilkes, 1965; Biggart, 1966a, b) and rabbits (Trench, Watson, Walker, Gardner and Green, 1966). In addition, bursal tissue enclosed in diffusion chambers has improved the antibody-producing capacity of bursectomized chickens (St. Pierre and Ackerman, 1965, 1966; Janković and Leskowitz, 1965). Other workers have failed to demonstrate any reconstitutive or protective effect of enclosed thymus or bursa or have failed to demonstrate any activity specific for these tissues (Huvos, Cali and Azar, 1966; Dent and Peterson, 1967). In most of the experiments, other than those of Osoba and Miller (1964) and Sherman (1967), the extent of immunological reconstitution has been slight and often unconvincing. Antibody titres and skin graft mean survival times rarely approach those in intact animals and many animals do not show any evidence of heightened immunological responsiveness. It is not known whether these failures simply reflect conditions within some chambers which are inimical

to cell survival. Further criticisms include the possible migration of cells through membranes with a large average pore diameter (Hays, 1964; Barclay, 1964) and the fact that it is technically not feasible to adequately test each diffusion chamber, for construction faults, prior to implantation.

Thymus tissue within chambers rapidly loses its lymphoid appearance but epithelial reticular cells appear to remain viable for many weeks or months (Grégoire, 1958; Osoba and Miller, 1963; Levey et al., 1963; Weiss and Miller, 1966; Wong et al., 1966; Biggart, 1966a; Trench et al., 1966; Schaller and Stevenson, 1967; Hays, 1967). The epithelial cytoreticulum in the normal mouse and rat thymus, and in cultured mouse thymus explants, displays histological, histochemical, and ultrastructural features which are in keeping with a secretory function (Arnesen, 1963; Clark, 1966, 1968; Shelton, 1966; Cherry, Eisenstein and Gluckmann, 1967). Supporting evidence for the existence of a thymic humoral factor(s), and the complicity of the epithelial component of the thymus, has come from two other sources. Allogeneic thymus grafts in adult-thymectomized, irradiated, and marrow-protected mice, and xenogeneic grafts in neonatally-thymectomized mice, failed to become lymphoidal (Dukor et al., 1965; Law, 1966b, c.f. Yunis, Martinez and Good, 1964) yet some of the hosts were able to reject tumour and skin allografts and respond to sheep erythrocytes by producing significant amounts of haemolytic antibodies (Leuchars et al., 1965; Law, 1966b; Feldman and Globerson, 1964; Miller et al., 1964). The epithelial cytoreticulum persisted for several weeks following grafting but was ultimately rejected (Dukor et al., 1965; Law, 1966b).

The immunological responsiveness of neonatally-thymectomized female mice was found to be improved following pregnancy (Osoba, 1965b, 1968a) and wasting disease was averted temporarily in a proportion of mated females (Elders, Parkham and Hughes, 1968). Transplacental

migration of foetal cells has been reported to be undetectable (Miller and Osoba, 1967). It is possible, however, that the pregnant mice, by virtue of their ability to maintain pregnancy, were a select group of animals in which the capacity to participate in immunological responses was greater, or was bound to improve more, than in the overall population of neonatally-thymectomized mice.

The autonomous lymphopoietic behaviour of the thymus and thymus grafts has been discussed previously (Section IA). The mitotic index of host-derived lymphocytes in thymus grafts reflects the origin of the thymus graft donor and presumably the activity of the persisting epithelial cytoreticulum. Thymus grafts, depleted of lymphocytes by irradiation (Miller et al., 1966) or by maintenance in vitro or in diffusion chambers (Reese and Israel, 1967; Hays, 1967), became infiltrated with lymphocytes on subsequent in vivo implantation and improved the immunological responsiveness of neonatally-thymectomized mice. Furthermore, some, but certainly not all (Vanderputte, 1967; Stutman, Yunis and Good, 1967), polyoma virus or chemically-induced non-lymphoidal thymic tumours may be reconstitutive when implanted into neonatally-thymectomized mice (Law, Dunn, Trainin and Levey, 1964; Stutman et al., 1967). It has yet to be resolved whether traffic of host lymphoid cells through the implanted tumour tissue is a necessary prerequisite of reconstitution. Recently, the rapid recovery of PHA responsiveness of lymphocytes from a patient with DiGeorge's syndrome following thymus grafting, has been used as evidence for a thymic humoral factor in immunogenesis (August, Rosen, Filler, Janeway, Markowski and Kay, 1968). The indirect evidence, obtained from the grafting experiments, supports the contention that the reticuloepithelial stroma of the thymus is intimately concerned with the lymphopoietic and immunological activity of the thymus.

Several groups have been engaged in work designed to extract and characterise thymic factors with lymphopoietic- or competence-inducing properties. Numerous reports indicate that heterologous

homologous and isologous thymus extracts may stimulate lymphoid cells or increase peripheral blood lymphocyte levels in normal mice (Metcalf, 1956, c.f. Duplan, Foschi and Manson, 1962; de Somer et al., 1963; Klein, Goldstein and White, 1965; Goldstein, Slater and White, 1966; Trainin, Burger and Kaye, 1967; Hand, Caster and Luckey, 1967). "Thymosin" (Goldstein et al., 1966), and the extract of Trainin et al. (1967), increased the incorporation of tritiated thymidine into DNA of lymphoid cells in vivo and in vitro but Berliner, Garzon, Lonngi and Dougherty (1967) found that fibroblasts in vitro also incorporated more tritiated thymidine when "thymosin" was added to the cultures.

In most of the experiments, extracts of thymus from sheep, calf and rabbit have been used and control preparations, when employed, have included lymph node and spleen extracts. The failure of the control extracts to induce lymphocytosis or promote thymidine incorporation into cells strongly suggests that the thymus extracts contain specific inducer substances. It is possible that these inducers are nothing more than potent foreign thymus antigens which are quantitatively and/or qualitatively different from antigenic material in extracts prepared from peripheral lymphoid tissues. The presence of antigens, apparently confined to the thymus environment in rats (Tallberg and Kosunen, 1966; Potworowski and Nairn, 1967) and mice (reviewed in Boyse, Miyazawa, Aoki and Old, 1968), has been well documented.

The contribution of foreign thymus-specific antigens becomes important in the interpretation of those experiments in which thymus extracts have been used to reconstitute the immunological reactivity of neonatally-thymectomized mice and adult-thymectomized, irradiated mice (Trainin and Linker-Israeli, 1967). Multiple injections of calf thymus extracts, but not calf muscle or kidney extracts, reduced the

mean survival time of allogeneic skin grafts and reduced the percentage of successful tumour transplants. Spleen cells from the reconstituted mice were able to mount GVH reactions in appropriate F_1 hybrid recipients. If thymus-specific antigens cross react with mouse histocompatibility antigens then the continuous stimulation imposed by the multiple injection regime may have sensitized the thymectomized recipient mice to antigens present on allogeneic cells. A precedent for the operation of such a mechanism may be the finding that multiple injections of sheep erythrocytes increase , to normal levels, the specific haemolytic and haemagglutinating antibody responses in neonatally-thymectomized mice (Takeya and Nomoto, 1967a). A search for "antithymus extract" antibodies in reconstituted mice does not seem to have been undertaken. It is equally feasible, of course, that antigens in extracts of nonthymic heterologous organs differ from thymic antigens in that they compete with foreign histocompatibility antigens for the relatively few immunocompetent cells presumed to be present in the tissues of immunologically defective mice.

Simple saline extracts of syngeneic thymus have not reversed the immunological deficiency in neonatally-thymectomized mice (Dalmasso et al., 1963; Miller, 1964a; Miller J.F.A.P., personal communication) but a reduced incidence of a fatal adenovirus infection (de Somer et al., 1963) and wasting disease (Trainin, Bejerano, Strahilevitch, Goldring and Small, 1966) has been recorded in neonatally-thymectomized mice injected with heterologous thymus extracts. Law and Agnew (1968) prepared thymus and spleen extracts, containing a few viable cells, from neonatal C57BL mice and injected them subcutaneously into syngeneic neonatally-thymectomized recipients. Spleen cells from the recipients of thymus extract, but not those from recipients of spleen extract, killed a large number of Balb/c mice when injected in the neonatal period. In contrast to the findings of Trainin et al. (1966) and Trainin and Linker-Israeli (1967), wasting

disease was not obviated and lymphopenia persisted. Small and Trainin (1967) injected neonatally-thymectomized mice with extracts of calf thymus and challenged them with sheep erythrocytes. The increase in splenic PFC and serum haemolysin titres was most unimpressive and kidney extracts, rather than nonthymic lymphoid tissue extracts, were used as control preparations.

In conclusion, it seems that no convincing evidence for a thymus specific competence-inducing factor, operating under physiological conditions, is as yet available. In all of the various systems, however, a substantial improvement has been achieved in a proportion of the immunologically deficient animals. Conceivably, factors may indeed be secreted by cells of the thymus epithelial cytoreticulum. There is a suggestion in the recent literature that thymic humoral factors are concerned with the ability of lymphocytes to respond to PHA, to mount GVH reactions and to participate in homograft reactions. The amelioration of defective antibody responses with thymus extracts has been far less impressive. If some thymus lymphocytes are immunologically competent, and are destined to leave the organ, then a relatively simple hypothesis may be formulated which embraces both cellular and humoral mechanisms of thymus activity in immunogenesis. Diffusable factors, elaborated by the epithelial cells may act locally on thymus lymphocytes and impart a strong proliferative stimulus to such cells and/or induce competence under conditions of proliferation, selection, differentiation and maturation. The mechanisms whereby competence is induced in thymus lymphocytes, and the nature of the events at the genomic level, must remain speculative (Jerne, 1967c). Alternatively, the factors may act at a distance and promote maturation of antigen-reactive faculties in peripheral cells which may or may not be thymus derived.

II. EXPERIMENTAL

A. Statement of the Problem and Purpose of the Experiments

At the time of commencing the experiments, several reports from various laboratories had clearly shown that young adult neonatally-thymectomized mice failed to respond in normal fashion to a primary injection of sheep erythrocytes. The peak number of splenic plaque-forming cells was usually much less than that in the spleen of intact mice of the same age. Reconstitution experiments, using thymus tissue enclosed in millipore diffusion chambers, had indicated that the thymus may exert its effect in immunogenesis partly by means of a humoral factor. To account for the decreased number of antibody-producing cells in thymectomized mice, two mechanisms of thymus activity had been proposed. Both hypotheses embraced the thymus humoral factor. The factor(s) could either act in concert with antigen to accelerate the proliferative and differentiative response of antigen-reactive cells (Metcalf and Brumby, 1966), or be responsible for the maturation of antigen-reactive cells from some precursor cell population (Miller et al., 1965). In the former case the number of antigen-reactive cells in the peripheral tissues of neonatally-thymectomized mice would not be expected to be very different from that in normal mice but the latter hypothesis infers that the number of such cells is reduced.

The initial experimental undertaking was to determine the number of antigen-reactive cells in peripheral lymphocyte populations of neonatally-thymectomized CBA mice. The recently described haemolytic focus assays for antigen-reactive cells seemed suitable for this purpose (Kennedy et al., 1965a; Playfair et al., 1965).

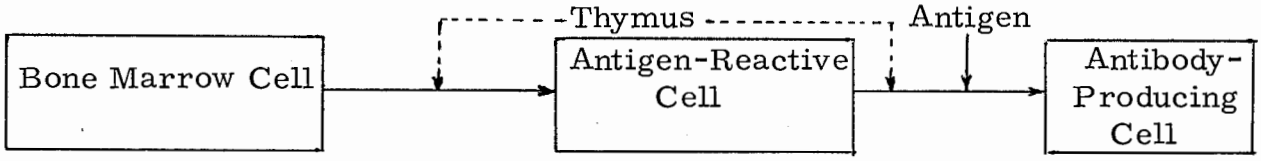
Gowans and his colleagues had shown that some thoracic duct small lymphocytes responded to histocompatibility antigens by undergoing blast transformation and that the population was involved in the initiation of certain antibody responses. The small lymphocyte seemed to be an outstanding candidate for the antigen-reactive cell and the popular notion had arisen that the long-lived recirculating lymphocyte responded to antigens, such as sheep erythrocytes, by undergoing blastoid transformation and subsequent proliferation and maturation into antibody-producing cell types. By injecting thoracic duct lymphocytes together with sheep erythrocytes into irradiated as well as neonatally-thymectomized mice, it was hoped that answers to the following questions would be forthcoming:-

(1) Are thoracic duct lymphocytes able to reconstitute neonatally-thymectomized mice with respect to plaque-forming cell production?

(2) Does the thoracic duct lymphocyte population contain antigen-reactive cells?

(3) If so, are the antigen-reactive cells able to proliferate and differentiate into antibody-producing progeny in the environment of the neonatally-thymectomized mouse and thus in the absence of the thymus?

It was thought that by enumerating antigen-reactive cells and studying their behaviour in neonatally-thymectomized mice, the primary site of action of the thymus would be pinpointed to one or other of two locations in the postulated genesis of the immune response:-



Depending on the results of these investigations, attention would be focussed on the nature of the cooperation between the thymus humoral factor and antigen or on the bone marrow and thymus as possible sources of antigen-reactive cells.

The reported ease with which thoracic duct fistulae could be established using cyanoacrylate adhesives (Boak and Woodruff, 1965) prompted attempts to quantitate the lymphocyte deficiency so clearly apparent in the peripheral lymphoid organs of neonatally-thymectomized mice. Previous work had indicated that the number of thoracic duct lymphocytes was indeed diminished after thymectomy at any age. The output determinations were, however, performed in anaesthetized rats, guinea pigs, and mice over short periods of time. Immunological defects following neonatal thymectomy were known to be more severe than those displayed by adult-thymectomized mice and a study of this difference, at the level of the recirculating lymphocyte population, was initiated. Finally, it was planned to extend these output studies to include neonatally-thymectomized mice injected with marked thymus cells and to determine whether these inocula contribute cells to the pool of recirculating lymphocytes.

B. Materials and Methods

(1) Animals

Mice of the highly inbred strains CBA, C57BL, CBA/T6T6 and the (CBA x C57BL)F_1 hybrid were used in the experiments. CBA and CBA/T6T6 mice were obtained originally from the Radiobiological Research Unit, Harwell, Didcot, Berkshire, England and the C57BL were obtained by Dr. R. Bradley, of the University of Melbourne, from Dr. L.W. Law, National Institutes of Health, Bethesda, Maryland, USA. The degree of inbreeding in the mice of the Hall Institute colonies was preserved by strict stemline brother-sister mating and the number of available mice was increased by two to three or, on rare occasions, four (but never more than four) generations of random matings. All mice were fed Barastoc dog pellets and tap water ad libitum with an occasional greenfeed supplement of cabbage leaves. Six to 10 weeks old CBA males which were weaned and separated at 5 weeks of age, were used in the vast majority of the experiments. In the case of neonatally-thymectomized or sham-operated mice, the limited availability of animals of any one age resulted in the use of both males and females as donors and/or recipients.

(2) Neonatal thymectomy

CBA mice were thymectomized within 24 to 36 hours of birth by the method devised originally by Miller (1960). After cooling for 5 minutes at -10°C, the neonates were placed in dorsal recumbency with a pad to arch the back and with the fore limbs secured laterally by rubber bands (Figure 1). Using a No. 11 scalpel blade, a 5 to 6 mm midline incision was made, commencing at the second sternebra and terminating 2 to 3 mm anterior to the sternal notch. The sternal notch was exposed by anteriorly retracting the posterior pole of the submaxillary salivary gland and a V-shaped portion of the sternum

isolated by cutting from the sternal notch to the region of the second costochondral junction on both sides. By grasping and elevating the point of the sternum the mediastinal and fascial strands lying ventral to the thymus lobes were torn away using forceps or scissors. After removing the triangular piece of sternum, the thymus lobes could be brought into view by pressure on the abdomen (Figure 1). Using a glass nozzle fitted to a water pump, the posterior poles of the thymus lobes were retracted and sucked anteriorly away from the auricles of the heart. The nozzle diameter was similar to the transverse diameter of each thymus lobe and the suction pressure was carefully adjusted to preserve the integrity of the capsule during the manipulation of each lobe. Under 2x magnification, the lobes were removed individually, care being taken to prevent disruption of the major blood vessels in the neck. Sham thymectomy involved removing portion of the sternum and the fascial strands but leaving the thymus in situ and untouched.

After thymectomy or sham operation, the mice were removed from the operating field and the wound closed with a horizontal mattress suture. The suture material was 4-0 black braided silk fitted to a round-bodied, non-cutting, atraumatic needle. After swabbing the wound free of blood, the mice were warmed under a 100 watt lamp and fostered on to Hall Institute outbred mothers when fully active and breathing normally. Some mice occasionally failed to commence breathing because of pneumothorax. Air could be expelled from the chest and the mice revived by the application of intermittent pressure to the thoracic cage and abdomen. The actual loss of mice at surgery was slight but losses due to cannibalism and maternal neglect were substantial. When the mice were 1 to 2 weeks old, oxytetracycline (Terramycin, Pfizer Pty Ltd., Sydney, Australia) at a dose level of 100 mgm per litre or, in the earliest experiments, penicillin G, at a dose level of 600,000 units per litre, was added to the drinking water each day.

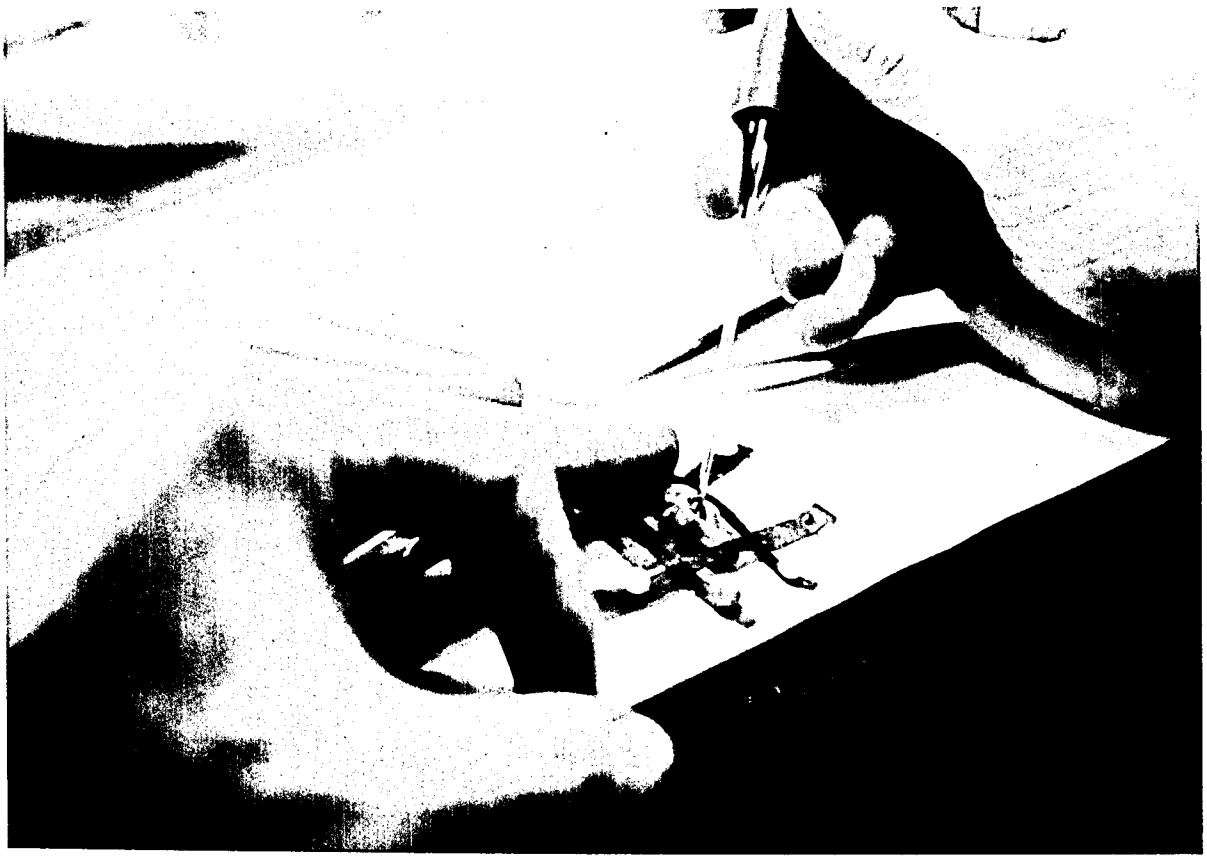


Figure 1. Extirpation of the thymus in newborn mice. The immobilized neonate is restrained in dorsal recumbency and finger pressure applied to the abdomen to force the heart and adjacent thymus lobes anteriorly. The posterior poles of the thymus lobes are teased away from the pericardium and the lobes aspirated individually into a glass nozzle attached to a water pump.

The presence or absence of residual thymus tissue was ascertained whenever neonatally-thymectomized mice were killed. The entire sternum was removed and the thymus area examined macroscopically in a bloodless field. Suspect thymus tissue was fixed in Bouin's fixative for 1 to 2 hours and transferred to 70% ethanol prior to embedding and serial cutting in the Histology Department of the Hall Institute. All mice with thymus remnants were discarded from the experiments. The number of such mice amounted to no more than 5 and the low incidence was ascribed to the ease with which thymectomy could be performed in CBA mice and to the policy of rejecting mice whenever the thymus capsule was broken or if the lobes could not be adequately visualized throughout the entire procedure.

(3) Adult thymectomy

The method used for removing the thymuses in adult mice was essentially the same as that used in neonates. Under ether anaesthesia each mouse was placed in dorsal recumbency with the four legs pulled laterally. A small pad of cotton wool was used to arch the back and, by means of a thread attached to the upper incisions, the head was pulled forward. The skin and sternal incisions were made with scissors but, in contrast to the neonatal operation, the piece of sternum removed was relatively small and was restricted to a 5 mm x 2 mm segment in the midline. The muscles and fascia lying ventral to the thymus were underrun with one cutting arm of the scissors and severed. The thymus lobes were forced to the surface of the wound by applying considerable pressure (with the fingers of the left hand) to the abdomen and hence the diaphragm. Each lobe was removed individually using more suction pressure than was the case in the neonatal operation.

In order to prevent pneumothorax and subsequent death of the mouse, it was absolutely essential to maintain a degree of pressure on the abdomen immediately after thymectomy. This pressure was discontinued only after the "back pad" had been removed and the front leg retractors released. Finally the skin incision was closed with two Michel clips, and the mice left to recover in a warm environment.

The large thymus lobes of 4 to 8 weeks old mice separated quite readily from the surrounding organs and tissues such as the heart, trachea, salivary gland and the pleural reflections adjacent to the apical lobes of the lungs. Losses at surgery amounted to no more than 5% of the operated mice and were usually the result of pneumothorax or excessive blood loss. They occurred more frequently in aged or stressed mice mainly because of the difficulty in separating the thymus lobes from the surrounding tissues.

(4) Preparation and injection of cell suspensions

Single cell suspensions of spleen, thymus and bone marrow were prepared in a manner similar to that described by Billingham and Brent (1959).

(i) Spleen cells. After stunning the mouse and dislocating the cervical vertebrae, the skin was swabbed with 70% ethanol and the spleen removed and placed on an 80 mesh, 0.005 gauge stainless steel sieve contained in a 9 cm plastic petri dish. Small curved forceps were used to disrupt the capsule and to tease the splenic contents through the sieve into single strength medium 199 (Tissue Culture medium 199 with 100 units penicillin and 100 μg streptomycin per ml, Commonwealth Serum Laboratories, Melbourne, Australia). Further disruption was achieved by aspiration of the filtrate through Pasteur pipettes. The residual tissue remaining on the sieve was teased gently and washed with medium 199 until only the fibrous stroma remained. The contents of the petri dish were transferred into sterile graduated

centrifuge tubes contained in a beaker of ice and water. The single cell suspension was spun in the cold at 400 to 450 g for 5 minutes, the supernatant discarded, and the pellet of cells resuspended in 5 to 10 ml of fresh medium 199. After respinning, the pellet was made up in medium 199 if the cells were to be used in the plaque-forming cell assays, or Dulbecco's phosphate buffered saline (PBS) if for injection into mice. Nucleated cell counts were then performed in a Neubauer haemocytometer, using 2% acetic acid as the diluent, and the volume of the suspension adjusted so that the required number of cells was contained in 0.1 to 0.2 ml. Single cell suspensions of cranial mesenteric, axillary and/or subiliac lymph nodes (Kawashima, Sugimura, Hwang and Kudo, 1964) were prepared and washed in a manner similar to that described above.

(ii) Thymus cells. Thymus lobes were removed from mice pinned in dorsal recumbency with the fore legs stretched laterally and the head extended. To ensure adequate exposure of the thymus area, the entire sternum was removed, care being taken to avoid cutting major blood vessels in the cervical and brachial regions. The thymus lobes were peeled and cut from the surrounding tissues and organs in a bloodless field, placed in a petri dish containing medium 199, and checked for fascial tags which might contain parathymic lymph nodes. Single cell suspensions of thymus were prepared as previously described for spleen but were always washed three times in medium 199. Clumps were removed each time the pellet was resuspended and the cells were finally resuspended in PBS.

(iii) Bone marrow cells. The long bones of the leg were removed, after cutting the skin from the metatarsus to the umbilicus, by tearing and cutting the adherent muscles and the ligamentous attachments to the acetabulum and tarsus. Residual musculature was scraped from the femurs and tibiae using a No. 21 scalpel blade and the shafts cut at each end through the epiphyses. By forcing a 25 or 26 G needle

into the severed ends, the marrow could be flushed out with medium 199 delivered from a 1 ml tuberculin syringe. Dissociation of the bone marrow plugs was achieved by aspiration through the needle and the cell suspension finally transferred to a sterile graduated centrifuge tube contained in ice and water. The entire procedure was performed in petri dishes containing cold medium 199 and resting on ice trays. The cells were washed once, resuspended in PBS, counted, and the volume for injection adjusted to 0.2 to 0.3 ml.

(iv) Erythrocyte suspensions. Jugular blood was obtained from a single sheep at weekly intervals. After collection into Alsever's solution the cells were allowed to stand at 4^{o}C for 7 days. Sheep erythrocytes (SRBC) were only used between 1 and 2 weeks after collection whether for immunization purposes, tolerance induction, or for the haemolytic antibody assays. Prior to use the cells were washed in normal saline until the supernatant was clear.

Horse blood cells were obtained from the Commonwealth Serum Laboratories, Melbourne, Australia, stored at 4^{o}C, and used within 5 weeks of collection. Care was taken to ensure that the same sample of blood was used for immunization and in the haemolytic antibody assays. The dose of sheep and horse erythrocytes injected into mice for immunization purposes was usually 0.1 ml of a 20% suspension and contained from 1 to 2 x 10^{8} erythrocytes.

(v) Parenteral injections in adult and newborn mice. Five to ten minutes before intravenous injection, adult mice were placed in a 37^{o} C incubator to dilate the lateral tail veins. With the mouse restrained in a narrow cylinder the veins were occluded at the base of the tail using the first and second fingers of the left hand. By rotating the tail 90^{o}, one vein could be brought into full view. All injections were made with a 25 or 26G needle attached to a 1 ml tuberculin syringe. Washed lymphoid cell suspensions for injection purposes were always finally resuspended in PBS and were contained in tubes

kept in an ice bath. When necessary, heterologous erythrocytes were added to, and mixed with, the cell suspension immediately prior to injection. The total injection volume varied from 0.1 to 0.6 ml. Large numbers of thymus cells were injected intravenously in a volume of from 0.4 to 0.6 ml. If, in addition, the cells were injected slowly over a period of 2 to 3 minutes, deaths in recipient mice from embolus formation could be obviated.

The intravenous injection of cells into newborn mice was performed according to the method described by Billingham and Brent (1956, 1959). The mice were held in the fingers of the left hand such that the skin over the right side of the head and neck was drawn taut and the right jugular vein compressed. The injection volume of 0.05 ml was expressed through a 30G needle into the orbital branch of the right anterior facial vein immediately aboral to the eye. When the needle was correctly placed, the inoculated medium could be seen, under 2-fold magnification, to blanch the venous plexus in the region of the injection site. Bleeding was profuse after the needle was removed and could be minimized by resting the ends of artery forceps bound in cotton wool on to the puncture wound. For identification purposes the toes were cut and the mice returned to the nest when bleeding had ceased.

Cells were injected intraperitoneally into mice younger than 1 week old by running a 30G needle subcutaneously from the anterior sternum to the area of the stomach and piercing the peritoneum. Injection volumes were restricted to 0.05 ml. Intraperitoneal injections in older mice were performed using a 26G needle to pierce the skin and abdominal muscles in the umbilical region. Subcutaneous injections were performed with an assistant holding the mouse by the tail and nape of the neck. The inoculum was deposited over the side of the thorax after underrunning the skin with a 26G needle for a distance of 1 to 2 cm.

(5) Thoracic duct cannulation and collection of thoracic duct cells

The technique used to establish a thoracic duct fistula was a modification of the method described originally by Boak and Woodruff (1965)and more recently by Morse and Riester (1967) and Mandel (1967). The use of cyanoacrylate tissue adhesives to secure polythene cannulae into position has greatly increased the operative success rate in mice. The short intraabdominal portion of the thoracic duct cannot be dissociated from the aorta, and this anatomical feature makes it difficult to affix cannulae into position by means of ligatures as described in the rat by Bollman, Cain and Grindlay (1948). Nevertheless, two reports have indicated that, with slight modification, the technique used in rats can be successful in mice (Shrewsbury, 1959; Gesner and Gowans, 1962a).

The most satisfactory anaesthetic agent was found to be Avertin (Avertin with amylene hydrate, Winthrop Laboratories, N.Y., at a dose level of 0.1 ml per 10gm body weight of a 2% solution in 10% ethanol) since, after intraperitoneal injection, the duration of surgical anaesthesia was restricted to about 10 minutes. Restrained mice were invariably able to run on the modified Bollman cages (Bollman, 1948) within 60 minutes of the induction of anaesthesia provided they were kept warm in the postoperative period. This short recovery phase and the return of muscular activity markedly increased the rate of lymph flow and assisted in the removal of clots from the cannula. Ten to twenty minutes prior to commencing the operation, 0.3 to 0.4 ml of cream or olive oil was fed by intraoesophageal instillation. The presence of chylomicrons in the lymph demarcated the thoracic duct throughout the course of the operation.

With the anaesthetised mouse in right lateral recumbency, the left, or uppermost, fore and hind legs were extended anteriorly and posteriorly with retractors. The hair was clipped from the left lateral

aspect of the thorax and abdomen extending posteriorly to the hip. This area was swabbed with 70% ethanol and a 2 cm skin incision made with scissors from the costal arch to the region of the tuber coxae immediately lateral to the left kidney and parallel to, and 5 mm from, the line of the transverse lumbar processes. An incision of similar size could then be made in the abdominal muscles and peritoneum. Four curved pins attached to rubber bands served as retractors to extend the edges of the wound and to display the spleen, kidney and portions of the intestines, stomach and liver (Figure 2). With blunt dissection using two cotton buds, the kidney and perirenal tissues were reflected ventrally away from the sub-lumbar muscles and the thoracic duct approached retroperitoneally. Locating the short abdominal portion of the duct involved careful disruption of tissues lying medial to the left adrenal gland and lateral to the abdominal aorta. By retracting the "floating" kidney ventrally with one cotton bud, considerable tension could be applied to the aorta and the attached thoracic duct. The left wall of the duct was then gently cleared of laterally-associated fascia, and the sublumbar muscles pushed dorsally, with the other cotton bud.

At this stage the cotton buds were removed from the abdominal cavity and the short arm of the J-shaped polythene cannula (Portex Boak Thoracic Cannulae for mice, Portland Plastics Limited, Hythe, Kent, England) cut at an angle about 0.5 cm distal to the 180^{o} curvature. Placing the cannula through the skin and muscles of the posterior paralumbar region was achieved by piercing these tissues with an 18 gauge needle, feeding the long arm of the cannula into the bevelled end of the needle within the abdominal cavity, and removing the needle. The point of emergence was high enough in the flank to prevent fouling of the cannula by movement of the left hind leg when the mouse was restrained on the exercise wheel. The long arm of the cannula was attached to a 25 gauge needle fitted to a 2 ml syringe filled with heparin

saline (100 units/ml). The syringe was placed in a plasticine mould so that it could be used to rotate the cannula within the abdominal cavity at will. The entire length of the cannula was then filled with the anticoagulant solution.

With the intraabdominal portion of the cannula lying slightly lateral to its definitive position, the thoracic duct was relocated and the lateral wall tensed by downward (ventral) pressure on the left kidney. A small, sharp pointed pair of jewellers forceps was used to pierce the wall between two small lumbar arteries and veins which lie adjacent to the duct. If tension was maintained whilst puncturing the duct, air was drawn in and the chylous lymph displaced. This manipulation was found to be important since it clearly defined the position of the hole and the width of the duct. The bevelled end of the cannula was then fed into the duct for a distance of approximately 2 mm (Figure 3) and the cotton bud pressure on the kidney relaxed. This method of insertion was shown to be far more positive than the usual practice of stabbing the duct with the pointed end of the cannula and reduced the risk of lacerating the duct or penetrating the right lateral wall which lies adjacent to the posterior vena cava.

Lymph either entered the tubing of its own accord or could be aspirated by slight extrusion of the plunger of the syringe. A small drop of tissue adhesive (Ethicon methyl or isobutyl 2-cyanoacrylate monomer, Ethicon Inc., Sommerville, N.J., U.S.A.) sealed the fistula and was allowed to polymerise in the area of union between duct and cannula for 1/2 to 1 minute. The abdominal contents were swabbed with a generous amount of warm physiological saline and the kidney returned to its preoperative position. Finally, the syringe was disengaged from the free end of the cannula and the wound and leg retractors removed.

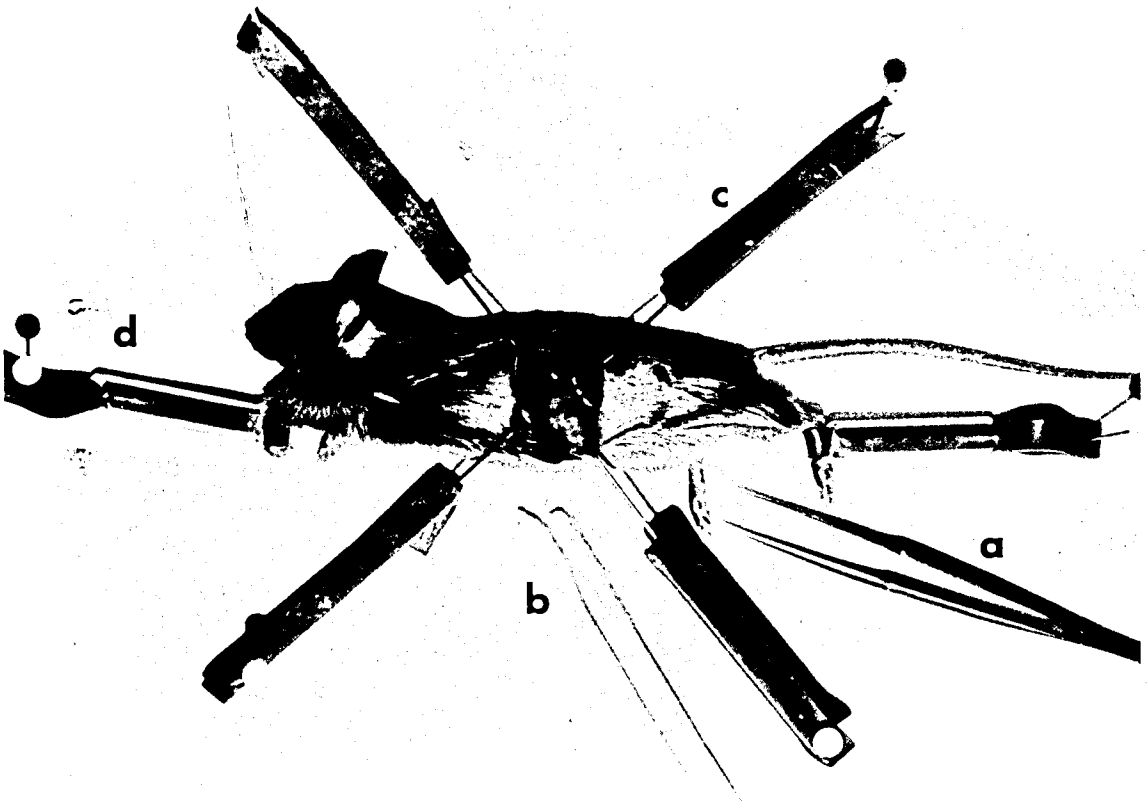


Figure 2. Anaesthetized mouse after laparotomy and extension of wound edges.

a, = pair of jewellers forceps;

b, = cotton buds;

c, = wound retractor;

d, = leg retractor.

The spleen, left kidney and portions of the large and small intestines are visible, within the limits of the wound.

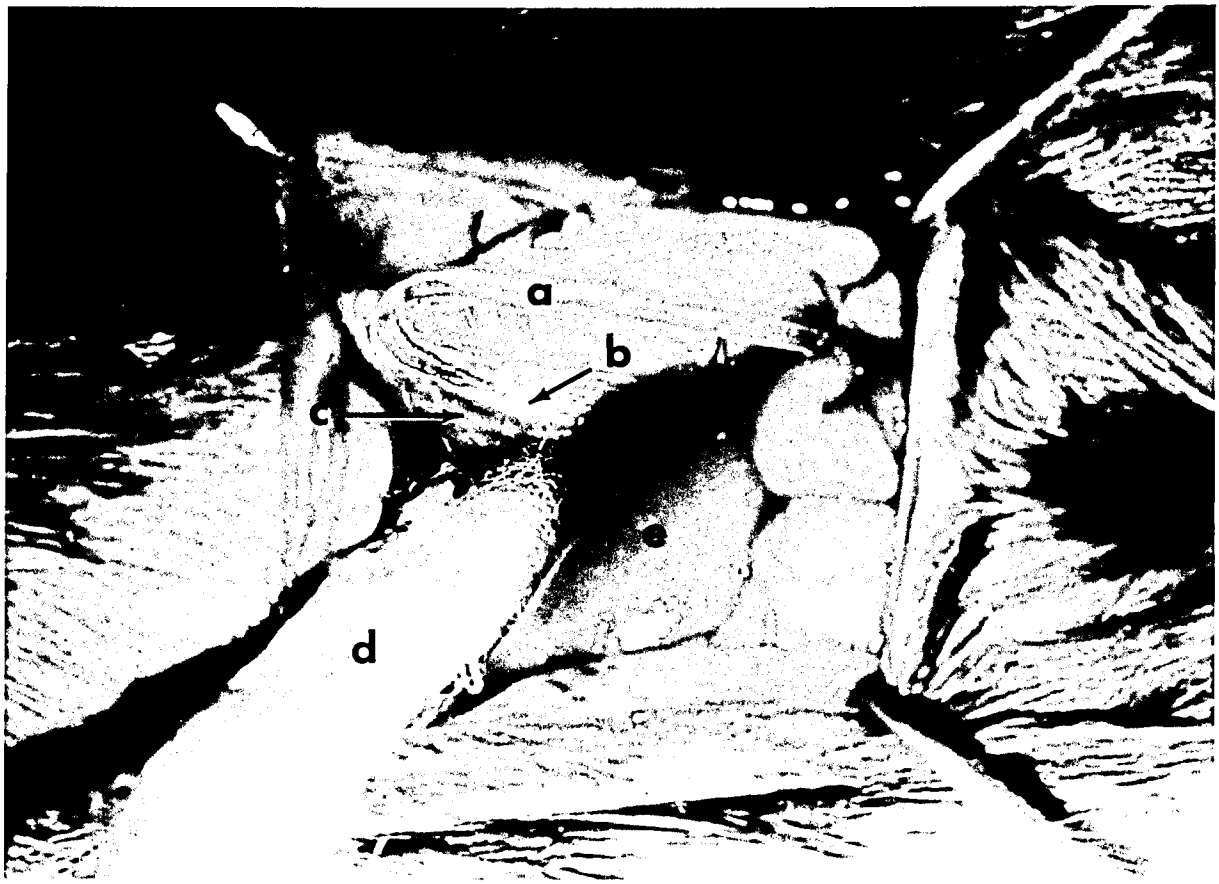


Figure 3. Site of insertion of polythene cannula into the short intraabdominal portion of the thoracic duct.

a, - long arm of cannula resting against the sub-lumbar muscles;

b, - thoracic duct filled with chylous lymph;

c, - intraabdominal aorta;

d, - cotton bud used to retract the left kidney (e), spleen and intestines, and to exert tension on the aorta and attached thoracic duct wall.

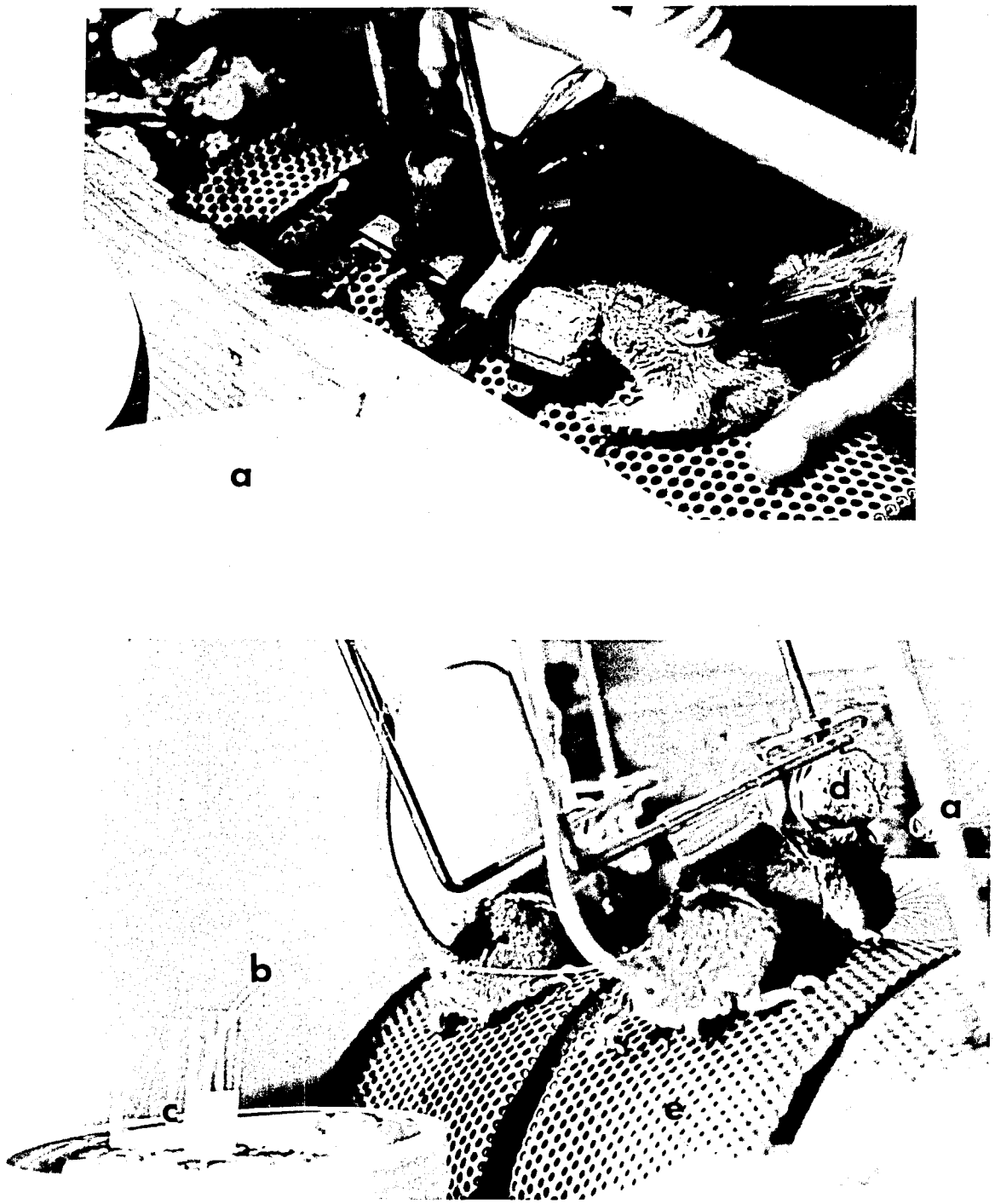


Figure 4. Restrained, unanaesthetized mice on modified Bollman cages after establishment of thoracic duct fistulae.

a, drinking bottle filled with glucose-saline and nozzle
b, polythene cannulae; of same;
c, collection tube in ice and water;
d, Barastoc dog pellet;
e, exercise wheel.

The muscle layers were sutured with silk (Deknatel Cardiovascular 4-0 black braided silk, J.A. Deknatel and Son. Inc., N.Y., U.S.A.) and the skin wound closed with 3 Michel clips and painted with collodion flexile. The entire operation could be completed in 3 to 4 minutes without magnification provided a powerful light source was available to illuminate the depths of the wound.

As a dressing, and for the purposes of restraint, the thorax and abdomen were bound in tape which was in turn secured to a rod. The mouse, with its tail fixed to the rod to prevent continual brushing of the cannula, was transferred to the modified Bollman exercise cages and allowed to recover (Figure 4). Collection into 10% normal mouse serum or foetal calf serum (Commonwealth Serum Laboratories, Melbourne, Australia) in PBS containing 50 to 100 units of preservative-free heparin (Evans Medical Limited, Liverpool, England) per ml, was commenced after the lymph had displaced the heparin-saline within the cannula. The siliconised collection tubes were immersed for the greater part of their length in vacuum flasks filled with ice and water (Figure 4).

Although 5% glucose saline and Barastoc dog pellets were available to the mice at all times, forced feeding with glucose saline (1 to 2 ml in 4 or 5 small doses) by intraoesophageal instillation was found necessary in many cases during the first 24 hours. The liquid intake fluctuated during the course of chronic thoracic duct drainage and was reflected in the volume of lymph collected. To obtain adequate numbers of lymphocytes or meaningful output data, constant attention to the cannulae was necessary to remove lymph clots. These clots commonly occurred during the first 24 hours when the mice showed a reluctance to drink. They could be removed readily by passing a fine pointed horse hair up and down the cannula. When obstruction involved the inaccessible short arm of the cannula, lymph flow could often be recommenced

by forcing the collection medium or heparin-saline a short distance into the cannula by means of a syringe. In some cases, 0.3 ml saline containing 4 units of heparin was injected subcutaneously immediately after the operation and 12 and 24 hours later. The drainage of lymph was not always immediate and at times commenced only after the mice became active on the wheels.

The great majority of inbred CBA and C57BL mice tolerated the anaesthetic, operation, and restraint and only 5 to 10% failed to drain from the outset. Failures in hybrid mice were slightly higher mainly because of their vigorous activity on the wheels which caused the rupture of small or large blood vessles adjacent to the cannula. When traces of blood were apparent, the mice were discarded or the collection of lymph recommenced when the contents of the cannula had cleared.

Mouse thoracic duct cells were never left for long periods in vitro even at 0 to 4° C. The viability of the cells, as measured by their capacity to exclude trypan blue or eosin dyes, was found to be reduced when the collection time exceeded 12 hours. Viability tests on mouse thoracic duct cells collected for 8 to 12 hours were performed on 6 occasions and the percentage of stained cells was never greater than 7%. Smears of thoracic duct cells were found to be unsatisfactory unless the cells were first spun out of 1 to 2 ml of foetal calf serum. The pellet was resuspended in the small volume of foetal calf serum remaining in the bottom of the tube after the supernatant had been discarded. Smears of cells on glass slide were stained with Wright's stain and differential cell counts and measurements of lymphocyte diameter performed under oil immersion using an ocular grid with micrometre graduations. For each thoracic duct cell suspension at least 1,000 cells in not more than 4 smears were examined and measured.

(6) Irradiation of mice and cells

CBA and (CBA x C57BL)F_1 hybrid mice received 800 rads or 900 rads whole body x-irradiation depending on the machine used. In the early experiments, mice received 900 rads at the Peter MacCallum Clinic. At the Hall Institute, all mice received 800 rads. In the former case, the Philips deep X-ray machine delivered a beam with a half value layer (HVL) of 1.0 mm Cu and was operated under conditions of 250 kV, 15mA, 0.25 mmCu and 1 mm Al filtration and a F.S.D. of 30 cms with full backscatter conditions. The dose rate at the treatment surface was 300 rads per minute and the dose at the central point in the treatment volume was taken as the dose delivered to the mice. Maximum deviations from this central dose were +14% and -8% throughout the treatment volume. The mice, contained in a perspex box, were irradiated in groups of eight by a single x-ray field.

The irradiation procedures, and the conditions under which the Hall Institute Philips RT 250 machine was operated, were similar to these described above. The x-ray beam had a HVL of 1.0 mm Cu, the F.S.D. was 50 cm and the absorbed dose rate 170 rads/minute. Thoracic duct cells and thymus cells were irradiated (1,000 rads) in cold 30 mm plastic dishes at a dose rate of 120 rads/minute. The cells were suspended in ice cold Eisen's balanced salt solution containing 10% foetal calf serum in a volume which completely filled the dish.

To obtain mortality data following large doses of x-irradiation, male and female CBA mice received 800 and 910 rads and deaths were recorded each day. Losses were first noticed at 7 days following 800 rads, and 100% were dead by 13 days (30 mice). 910 rads killed 15/15 mice within 11 days. All irradiated mice were fed oxytetracycline (100 mgm/litre) in the drinking water.

(7) Haemolysin plaque-forming cell assays

Two techniques were used to enumerate cells in a single cell suspension which form plaques of haemolysis when incubated with erythrocytes and complement. The more extensively used technique was that described originally by Jerne et al. (1963) in which cell suspensions containing plaque-forming cells (PFC) are suspended in agar together with the appropriate erythrocytes. Plaques are developed by adding complement after a period of incubation. When assaying for the number of anti-horse erythrocyte plaque-forming cells, the agar-free method of Cunningham and Szenberg (1968) was used invariably.

(i) The Jerne PFC assay

Preparation of agar base plates. A 2.8% Difco agar solution in distilled water was autoclaved for 10 minutes at 5 lbs pressure and cooled to 60°C. Eagle's medium (Eagle's (Basal) Medium 10x concentrate with 500 mcg streptomycin and 500 units penicillin per ml, Commonwealth Serum Laboratories, Melbourne, Australia) at 2x concentration was heated to 45° C in a water bath and 5.5% sodium bicarbonate added in a ratio of 1 : 100 (v/v). Equal volumes of Eagle's medium and molten agar were mixed thoroughly by swirling in a flask and the mixture left for 10 minutes at 45°C. Bubbles were removed from the surface of the mixture at this stage. Finally, a volume of 8 to 10 ml of the Eagle's agar mixture was poured into 9 cm plastic petri dishes and allowed to solidify. These agar base plates were stored at 4°C and used from 1 to 7 days after preparation.

Preparation of soft agar. A 1.4% Difco agar solution in distilled water was autoclaved and decanted, with a preheated measuring cylinder, into preheated 30 ml bottles. These bottles, which usually contained 12.5 ml agar, were autoclaved for 10 minutes at 5 lbs pressure and allowed to cool to room temperature.

Preparation of dextran. 100 mgm DEAE dextran (Pharmacia, Uppsala, Sweden) was dissolved in 10 ml distilled water with vigorous and prolonged agitation. The dextran solution was used within one week of preparation.

Preparation of overlay mixture. A 12.5 ml sterile aliquot of soft agar was heated to 100°C and allowed to cool in a water bath to 45°C. Two and a half mls of 10x concentrated Eagle's medium was made up to 12 mls with distilled water and warmed to 45°C after the addition of 0.5 mls of 5.5% sodium bicarbonate. The 12.5 ml volumes of Eagle's and soft agar were mixed and 1.9 ml aliquots added to 0.1 ml dextran in clean tubes heated to 45°C.

Plating technique. After removing condensations, the agar base plates were placed in a 37°C incubator for approximately 30 minutes. To the tubes containing dextran and Eagle's agar, volumes of 0.1 ml of 30% sheep erythrocytes in saline and 0.1 ml of the cell suspension containing PFC, were added and mixed by inverting the tube once after placing a piece of "Parafilm" over the top of the tube. The mixed contents of the tube were poured onto the preheated agar base plates, spread by revolving and tilting the plate, and allowed to solidify for at least 10 minutes. Duplicate plates were prepared for each cell sample.

Development of plaques. The plates were incubated for 1 to 2 hours at 37° C after which time 2 ml of a 1 : 10 dilution of guinea pig serum in saline was poured on to each plate. Bubbles were avoided and care was taken to ensure that the whole plate was covered with complement. After a further incubation period of 0.5 to 1 hour the complement was poured off and the plates washed with cold normal saline. The plaques were counted under a dissecting microscope on the same day or after storing the plates overnight at 4°C. Finally, the number of PFC per spleen was calculated by multiplying the average number of plaques per plate by the appropriate dilution factor.

Technical failures were rarely encountered but when faults did occur they could usually be ascribed to mosaic formation (moving the plates during solidification of the soft agar overlay), separation of the top layer (agar base layer too fresh, dilute or not prewarmed), appearance of flakes (unclean glassware) or the presence of unmelted agar in the overlay.

(ii) The Cunningham PFC assay.

The agar-free technique for the detection and enumeration of PFC has several advantages over that described by Jerne et al. It is simpler and less time consuming and, because horse erythrocytes do not spread evenly in agar, the technique can be used in anti-horse erythrocyte plaque-forming cell assays.

Preparation of slide chambers. Glass slides (75 x 25 mm) were washed in RBS and rinsed in tap and distilled water. Three pieces of "double sided" 3M scotch tape No. 410 were laid across the ends and middle of the slide. Another slide of similar size was pressed firmly on to the taped slide forming two chambers of similar volume (Figure 5).

Filling of chambers and development of plaques. Small tubes or the wells of haemagglutination trays (Figure 5) were used to prepare a mixture of 0.1 ml of spleen cells in medium 199, 0.01 ml of undiluted guinea pig serum, 0.01 ml of 25% erythrocytes in normal saline, and 0.04 ml of 2.5% erythrocytes in medium 199. This mixture was drawn up into a Pasteur pipette and expressed into the chambers which were "topped up" when necessary with 2.5% erythrocytes (Figure 5). The edges of the slide chamber were sealed with molten paraffin-"Vaseline" (1 : 1) which was allowed to solidify. After incubation at 37°C for 30 to 45 minutes, the plaques were counted under a dissecting microscope. The only disadvantage of the method is that the plaques are unstable. The chambers must therefore be handled with care after incubation and the plaques counted immediately.

(8) The haemolytic focus assay

Kennedy et al. (1965a) and Playfair et al. (1965) independently described similar techniques for the detection of discrete areas of haemolysis in the spleens of irradiated mice injected with lymphoid cells and sheep erythrocytes (Section IB). The method developed in this laboratory

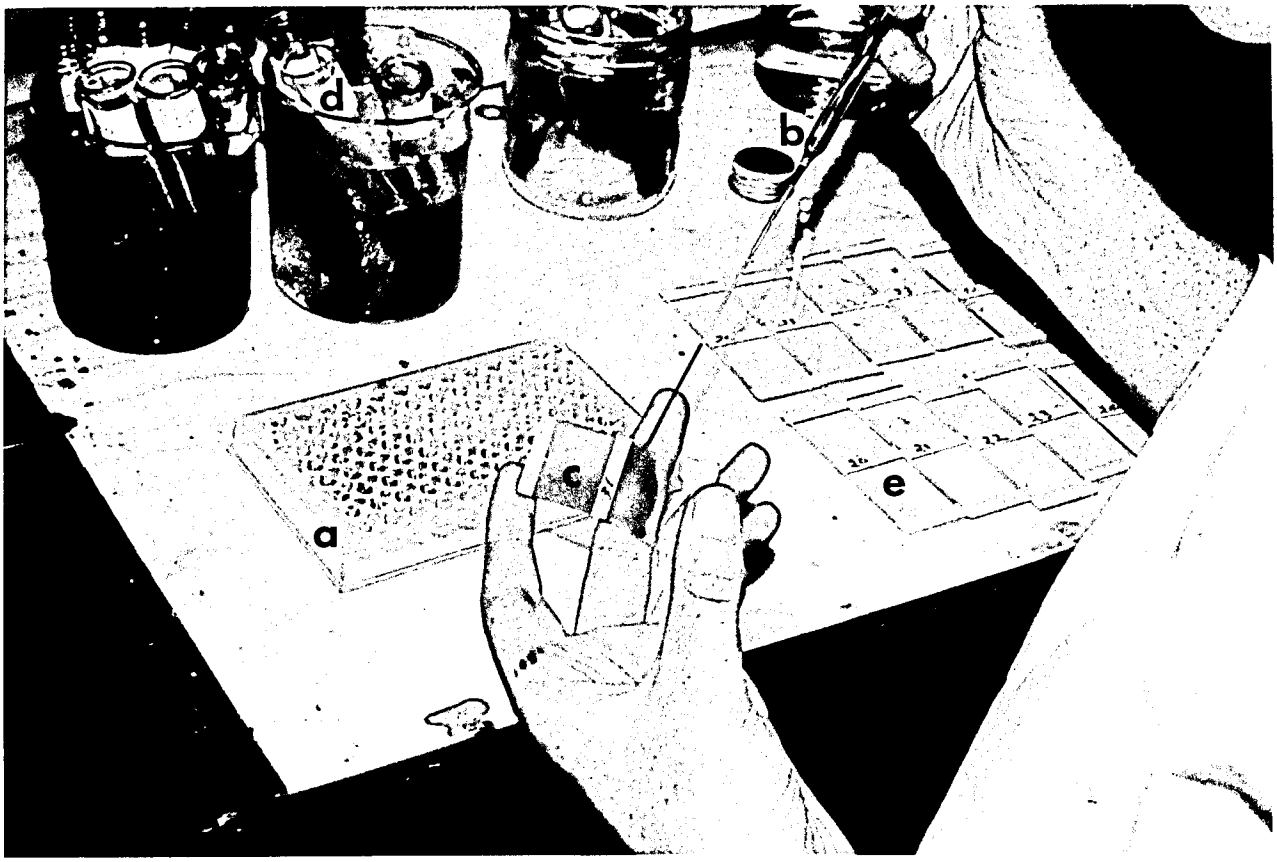


Figure 5. The technique used for filling slide chambers in the Cunningham plaque-forming cell assay. A mixture of spleen cells, heterologous erythrocytes and complement is prepared in the well of a plastic tray (a), taken up with a pipette (b), and expelled into the chamber (c). The open edges of the chamber are sealed with paraffin-"Vaseline" and the plaques developed over a 45 minute incubation period.
(d), spleen cell suspensions in graduated centrifuge tubes contained in ice and water; (e), numbered slide chambers ready for use.

was a modification of both techniques. Spleens were removed from mice and placed on the stage of a tissue chopper (McIlwain tissue chopper, Mickle Laboratory Engineering Co., Mill Works, Gomshall, Surrey, England) and moistened with phosphate buffered saline pH 7.3. 250 μ sections were cut and sequentially transferred with jewellers forceps to regular agar base plates cooled to 4^{o}C. Much care was taken to ensure that the sequential order of the spleen slices was maintained. The plates were warmed to 37^{o}C prior to pouring the Eagle's soft agar overlay, prepared as for the Jerne assay. A volume of 3.8 ml was added to the tubes containing 0.2 ml DEAE dextran, and to this was added 0.2 ml of 20% sheep erythrocytes. The tubes were inverted to mix the contents and the mixture poured over the spleen slices on the agar plates. When the top layer had solidified the plates were incubated at 37^{o}C for 1.5 to 2 hours. After the addition of complement (1 : 10 guinea pig serum) and further incubation for 1 hour, the plates were washed with saline and cooled to 4^{o}C.

Haemolytic foci could not be counted accurately unless the red cells were stained with a benzidine stain (Jerne et al., 1963). Two and a half ml of a mixture of 1 part 5% hydrogen peroxide to 9 parts benzidine (0.5 gm in 12.5% acetic acid) at 4^{o}C was added to the plates and allowed to stand for 1 to 2 minutes. After washing the stain from the plates with normal saline, the number of haemolytic foci was determined with the aid of a dissecting microscope. True haemolysis could be readily differentiated from the yellowish-green discolouration around many of the slices provided the plates were examined immediately after staining the red cells. The majority of haemolytic areas were apparent around the edges of the "active" slices but occasionally an area was detected in the top layer overlying the slice. The discolouration, which was more apparent around sections cut from spleens undergoing myeloid regeneration, could be minimised by absorbing the phosphate buffered saline and tissue juices from the cut spleens prior to placing the sections on to the agar plates. Technical difficulties in the plating technique could

be expected if attention was not given to the precautions outlined in the description of the Jerne PFC assay.

(9) Cyclophosphamide-induced tolerance to heterologous erythrocytes

The method used to induce immunological tolerance to heterologous erythrocytes was that described by Dietrich and Dukor (1967). CBA male mice were injected intraperitoneally, when 7 to 8 weeks old, with 0.5 ml packed sheep or horse erythrocytes. Cyclophosphamide (Endoxan Asta, Charles McDonald, Caringbah, N.S.W., Australia), at a dose level of 1 mgm per 10 gm body weight, was injected subcutaneously 24 hours later. Mice were injected in groups of 10, the total cyclophosphamide for the group having been dissolved in phosphate buffered saline pH 7.3 immediately prior to commencing the injections. Control mice did not receive erythrocytes and were injected with cyclophosphamide only. The reactivity of mice from both groups was tested 23 to 28 days later by challenging intravenously with 0.1 ml of a 20% suspension of either sheep or horse erythrocytes or a mixture of both. The number of PFC appearing in the spleen was determined 4 to 5 days after erythrocyte challenge. Thoracic duct cells were collected from nonchallenged mice at 4 weeks after cyclophosphamide administration. Thoracic duct fistulae were maintained for 16 to 24 hours and all donor mice were challenged with the appropriate erythrocytes 24 hours after closing the fistula and removing the mice from the exercise wheels.

(10) Skin grafting

The half-thickness, "open-style" skin grafting technique described by Billingham and Medawar (1951) was used throughout. Donor mice were selected after ensuring that a cycle of new hair growth did not involve the area of dorsal skin to be taken. Mice were killed, pinned in ventral recumbency, and the dorsum shaved and swabbed with 70% ethanol. Using small, nontoothed forceps the skin was raised into a cone and a piece removed with small curved scissors. The area of the

skin pieces was approximately 1 square cm and 8 to 12 pieces were obtained from each donor mouse. The subcutaneous fascia was scraped from the dermis and, with the epidermis uppermost, the pieces of donor skin were stored in a cooled, sterile, glass petri dish containing a filter paper moistened with normal saline.

Recipient mice were anaesthetised with ether and restrained in ventral recumbency with leg retractors extending the four legs laterally. The clipped area of skin over the lateral aspect of the thorax was swabbed with alcohol and tensed by pinching the skin on the contralateral side. A portion of dermis and epidermis, measuring in excess of 1 square cm, was removed using small, sharp, curved scissors, care being taken to preserve the panniculus adiposus and the contained blood vessels. This was effected most satisfactorily by placing the scissors flat on the skin and removing small segments with each cut.

A piece of donor skin of suitable size was placed on the graft bed such that overlap with recipient epidermis did not occur. Two sterilized squares of rice papers were next selected, one was placed over the graft site and the other on the adhesive surface of a 30 to 40 cm strip of sellotape. The edges of this strip were underrun to prevent abrasion of the axillae and abdomen when subsequently placed, as a dressing, around the thorax of the mouse. The rice paper adhered to, and covered, the graft and limits of the graft bed, and ipso facto provided protection to the site and prevented side-slip of the graft. With the fore legs pulled anteriorly, the head of the mouse was elevated and the sellotape strip placed under the chest. By folding the strip such that the two squares of rice paper were superimposed, the dressing was tightly wrapped around the thorax. Care was taken to ensure that the only pressure exerted on the graft was perpendicular to its surface. Tensions in any other direction often led to side-slip with the graft overlying a portion of the recipient epidermis. The grafted mice were

allowed to recover under a warming lamp and were carefully observed to ensure that respiration was not impeded.

Six to eight days after placing the graft the dressing was removed under ether anaesthesia. Grafts were examined for signs of rejection, and the state of the homograft recorded, every 12 to 24 hours. Technical failures were infrequent provided much attention was paid to surgical sterility and care was taken not to disturb the graft site when placing or removing the dressing.

(11) Preparation of isoantisera

Antisera were raised by injecting, at 2 to 4 weekly intervals, CBA and C57BL mice with 20 to 100 million cells from pooled spleen, thymus and mesenteric lymph nodes obtained from 6 to 10 weeks old C57BL and CBA mice, respectively. The intraperitoneal injections were commenced at 6 weeks of age and the number varied from 3 to 6. Each mouse received an appropriate allogeneic skin graft between the second and third injections. The majority of mice were kill-bled 7 to 10 days after the last of the immunizing injections. Other mice were bled from the eye and received another lymphoid cell injection up to 4 weeks later. These mice were kill-bled 6 to 9 days later.

All antisera and normal CBA and C57BL sera were inactivated at 56^{o}C for 30 minutes and stored at -20^{o}C in aliquots of approximately 0.5 ml.

(12) Incubation of PFC with isoantisera

In order to develop a technique for the inhibition of PFC and to test the efficacy of the isoantisera, normal CBA and C57BL mice were immunized with sheep erythrocytes and spleen cell preparations were prepared 4 days later. 0.15 ml aliquots of spleen cells, containing between 100 and 1,000 PFC were suspended in various media and incubated at 37^{o}C with undiluted anti-CBA, anti-C57BL, normal CBA

or normal C57BL serum in the presence or absence of complement. The cells were spun in the cold after 30 minutes incubation, washed once, plated onto regular Jerne agar plates, and the plaques developed in the manner previously described. Maximum inhibition of PFC with specific isoantisera, coupled with minimum losses when PFC were incubated with nonspecific antisera or normal mouse sera, were obtained by using a supporting medium of 10% foetal calf serum in Eisen's balanced salt solution and a ratio of spleen cells: serum : complement of 3 : 1 : 1 (v/v/v). The inclusion of complement (undiluted guinea pig serum) was found to be essential.

During the course of the experiments using isoantisera, a technique for the inhibition of PFC and the determination of inhibitory antibody titres appeared in the literature (Harris, Harris and Ogburn, 1967). The method used by these investigators was almost identical to that developed in this laboratory.

(13) Chromosome analysis of spleen cell suspensions

The technique used to prepare chromosome spreads of spleen cells was that described originally by Ford (1966b). Ninety to 105 minutes before death, the mice were injected intraperitoneally with 4μg Colcemid (Ciba) per gm body weight. Spleens were disrupted individually in 1% sodium citrate and the single cell suspension left in small Dreyer tubes for 15 minutes. After gentle centrifugation (circa 25 g), the hypotonic citrate solution was removed with a fine bore pipette and the pellet of cells loosened by flicking the end of the tube with the fore finger. A single drop of a freshly prepared solution of ethyl alcohol : glacial acetic acid (3 : 1, v/v) was run down the side of the tube. Immediately after contact between the fixative and the cells, the bottom of the tube was flicked for several seconds. Further drops were then added with constant but gentle agitation of the tube. At times the suspension was very gently aspirated through a pipette, care

being taken to avoid bubbles. The tube was then filled with the fixative and allowed to stand for 15 minutes.

After moderate centrifugation, the fixative was removed and the pellet resuspended in a small volume of 60% acetic acid. Four droplets of this cloudy suspension were added to a carefully cleaned slide which was then held over the tip of a small gas flame. When the volume of the drops had decreased by a factor of about 2 or 3, heating was discontinued and the slide waved about vigorously. The heating and shaking were repeated until the slide was dry. Finally, the cells were stained with 1 : 2 May-Grünwald (2 minutes) and 1 : 10 Geimsa (7 minutes) stains, coverslips mounted using DPX, and the number of mitotic figures per 2,000 cells counted under high power magnification (x312). The presence or absence of marker chromosomes in suitably spread mitotic metaphase preparations was ascertained, and chromosome counts performed, under oil immersion (x1250).

(14) Statistical analyses

Each set of observations was summarized using the statistics arithmetic mean ($\bar{x}$) and the standard error of the mean (SE). The following computing formulae for the estimation of the variance (S^2) and SE were used:-

$$S^2 = \frac{\sum_{i=1}^{n} x_i^2 - \frac{\left(\sum_{i=1}^{n} x_i\right)^2}{n}}{n - 1} \qquad \text{and SE} = \sqrt{\frac{S^2}{n}}$$

where x_i is the numerical value obtained from the i^{th} observation and n is the number of observations.

When the value of the ratio of the variances of 2 groups (A+B) was less than the value in the F-table corresponding to the 5% probability level, a standard 2-tailed Student's "t" test for small group sizes was used in the analysis of the data:

$$t = \frac{\bar{x}_A - \bar{x}_B}{\sqrt{\frac{SS_A + SS_B}{n_A + n_B - 2} \times \frac{n_A + n_B}{n_A n_B}}}$$

where SS_A and SS_B are the sums of squares of deviations about the means in group A and B, n_A and $\bar{x}_A$ are the number and mean of the observations in group A, and n_B and $\bar{x}_B$ are the number and mean of the observations in group B, respectively.

A modified "t" test, which had the overall effect of reducing the number of degrees of freedom, was used when the value of the variance ratio was greater than the value in the F-table corresponding to the 5% probability level (Smith, 1936; Bailey, 1959):

$$t_{df} = \frac{\bar{x}_A - \bar{x}_B}{\sqrt{\frac{S_A^2}{n_A} + \frac{S_B^2}{n_B}}}$$

where S_A^2 is the variance of group A, S_B^2 is the variance of group B and df is the number of degrees of freedom. This latter value was obtained by using the following formulae:-

$$\frac{1}{df} = \frac{C^2}{n_A - 1} + \frac{(1 - C)^2}{n_B - 1}$$

$$C = \frac{\frac{S_A^2}{n_A}}{\frac{S_A^2}{n_A} + \frac{S_B^2}{n_B}}$$

In the comparison of the means of any two groups of observations, a significance level of 0.05 was chosen.

C. Results

(1) Effect of thymectomy on the number of cells in the thoracic duct lymph

(i) Output of cells from the thoracic duct in normal mice.

Thoracic duct fistulae were established in 8 weeks old CBA male mice and the output of cells and lymph measured every 6 to 12 hours over a 10 day drainage period. No attempt was made to reinfuse the mice with cell-free lymph or nutrient solutions during the course of chronic thoracic duct drainage. The results, shown in Figure 6 and expressed as the output per day indicate that there can be no correlation between the number of cells and the volume of lymph drained on any one day. Whereas the output of cells decreased from an average of 91.7 million on day 1 to 25 million on day 5 and then remained constant between days 6 and 10 at approximately 10 million per day, the volume of lymph increased to a maximum of 19.2 ml on day 5 after which time it fell to 5.6 ml and increased progressively thereafter. The impression was gained from observing the mice that the output of lymph simply reflected the volume of glucose saline consumed. The average total number of cells drained in 10 days was 276.3 million of which 139 million (43%) had emerged in the first 48 hours. In output studies involving thymectomized mice (vide infra), the period of drainage was, in general, restricted to 48 hours.

The results of differential cell counts, performed on air dried smears of thoracic duct cells collected at various times, are shown in Table 1, group 1. 96.3% of the cells emerging from the fistulae on the first day were small lymphocytes (cell diameter $< 8\mu$) and this figure had fallen to 79% by day 9. The proportion of medium lymphocytes (cell diameter 8 to 10μ) and large lymphocytes (cell diameter $> 10\mu$) increased from 3 to 18.6% in this time and the relative number of other cells, classified as smudges, unidentifiable cells and polymorphonuclear leucocytes, also increased, albeit slightly. From the data in

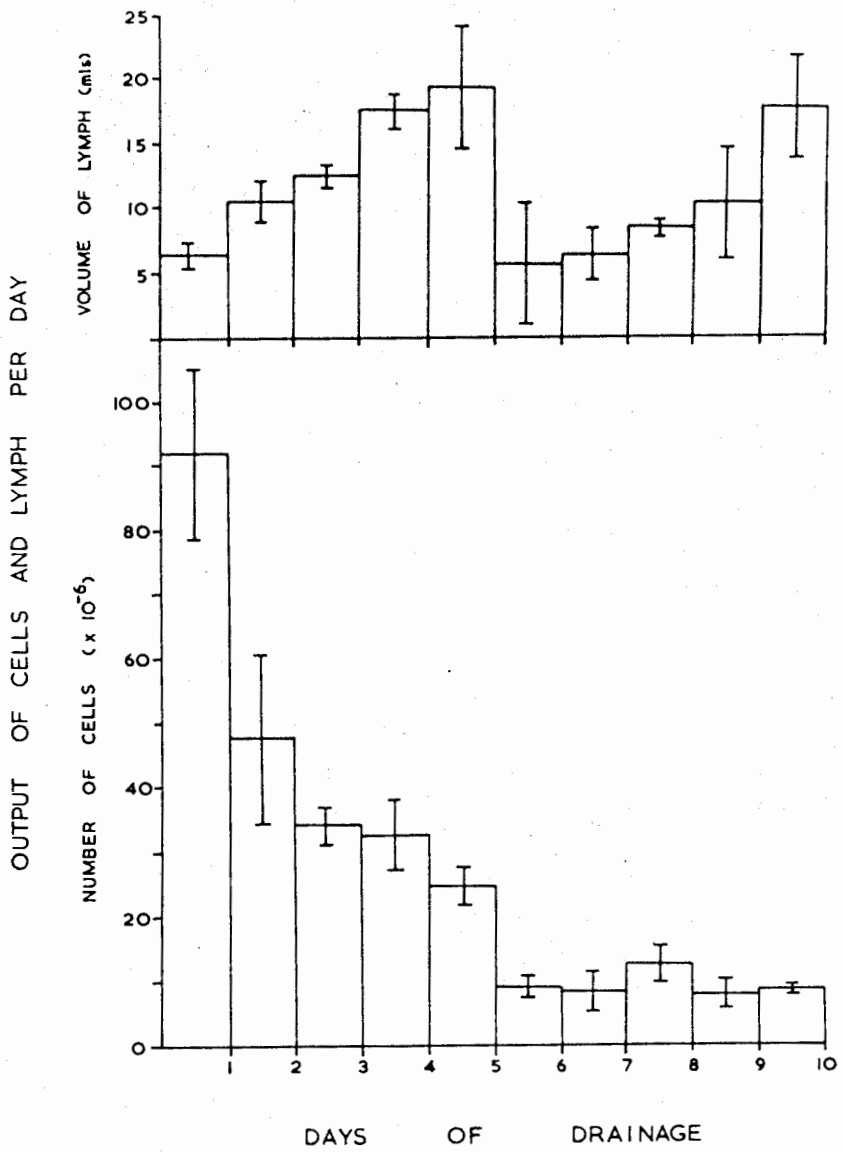


Figure 6. Daily output of cells and lymph from the thoracic duct of normal 8 weeks old CBA mice. Each block in the histograms from day 1 to 7 represents the average output from 3 mice and from 8 to 10 days, the average output from 2 mice. The vertical bars indicate the limits of two standard errors of each mean.

Table 1

Differential Counts of Cells in Thoracic Duct Lymph from Intact and Thymectomized CBA Mice

Group	Operation	Age at cannulation (weeks)	Hours of drainage	Differential cell counts[*]		
				% lymphocytes		% neutrophils, smudges and others unidentifiable
				Small	Medium and large	
1	Unoperated	8	0 - 6	96.3	3.5	0.2
			70 - 74	91.3	6.8	1.9
			98 - 102	85.5	10.5	4.0
			192 - 198	79.0	18.6	2.4
2	Neonatal thymectomy	5 - 6	0 - 6	66.5	25.7	7.8
			24-36	68.5	22.9	8.6
3	Sham neonatal thymectomy	5 - 6	0 - 6	93.3	3.7	3.0
			24-36	91.3	6.3	2.4
4	Thymectomy at 7 weeks of age	13	36-48	91.7	5.4	2.9
		43	36-48	83.4	14.4	2.2
		102	36-48	74.5	20.1	5.4
5	Sham thymectomy at 7 weeks of age	13	36-48	96.0	3.4	0.6
		43	36-48	87.8	10.6	2.6
		102	36-48	90.0	8.8	1.2
6	Thymectomy at 6 weeks of age, 900 rads and 10^7 marrow cells at 8 weeks	12	12-24	64.6	19.3	16.1
		16	12-24	80.3	16.9	2.8
7	Sham thymectomy at 6 weeks of age, 900 rads and 10^7 marrow cells at 8 weeks of age	12	12-24	75.3	16.3	8.4
		16	12-24	84.9	11.6	3.5

[*] 1,000 to 12,000 cells counted on smears of pooled samples from 2 - 8 mice. Small lymphocyte with cell diameter $< 8\mu$, medium and large lymphocytes with cell diameters $> 8\mu$.

Figure 6 and Table 1, group 1, it can be calculated that the absolute number of medium and large lymphocytes decreased from 3.2 million on day 1 to 1.5 million on day 9. This 53% reduction contrasts with the 92% reduction in the absolute number of small lymphocytes drained on day 9 as against the number emerging on day 1.

During the course of investigations into the capacity of thoracic duct cells to transfer immunological reactivity adoptively, it became necessary to look for anti-sheep erythrocyte haemolysin plaque-forming cells (PFC) in the thoracic duct lymph of normal mice. In three experiments, 10 to 25 million thoracic duct cells collected over 6 hour periods from normal mice, were assayed for their content of PFC. PFC were not detected in this population of cells. Eight weeks old CBA mice were then injected with 2×10^8 sheep erythrocytes by intraperitoneal injection and divided into 3 groups of 3 animals each. These groups of mice were cannulated on either day 3, 4 or 5 after immunization. Cells were collected every 8 hours and the number of PFC determined. The results of this experiment, performed in collaboration with Dr. T.J. Barclay, are shown in Figure 7. The time of cannulation did not alter the time of appearance of PFC in the thoracic duct; these cells appeared 4 to 6 days after sheep erythrocyte challenge in a well defined pulse. The peak number of PFC per million thoracic duct cells in the group of mice cannulated on day 3 was approximately three times higher than that in the group cannulated on day 5. From the data in Figure 6, the total output of cells in the former group could be expected to have fallen by a similar factor on the third day of drainage. Although total cell counts were not performed, and the effect of sheep erythrocyte inoculation on the output of thoracic duct cells 3 to 7 days later is unknown, it is unlikely that the absolute number of emergent PFC was very different in any of the 3 groups of mice.

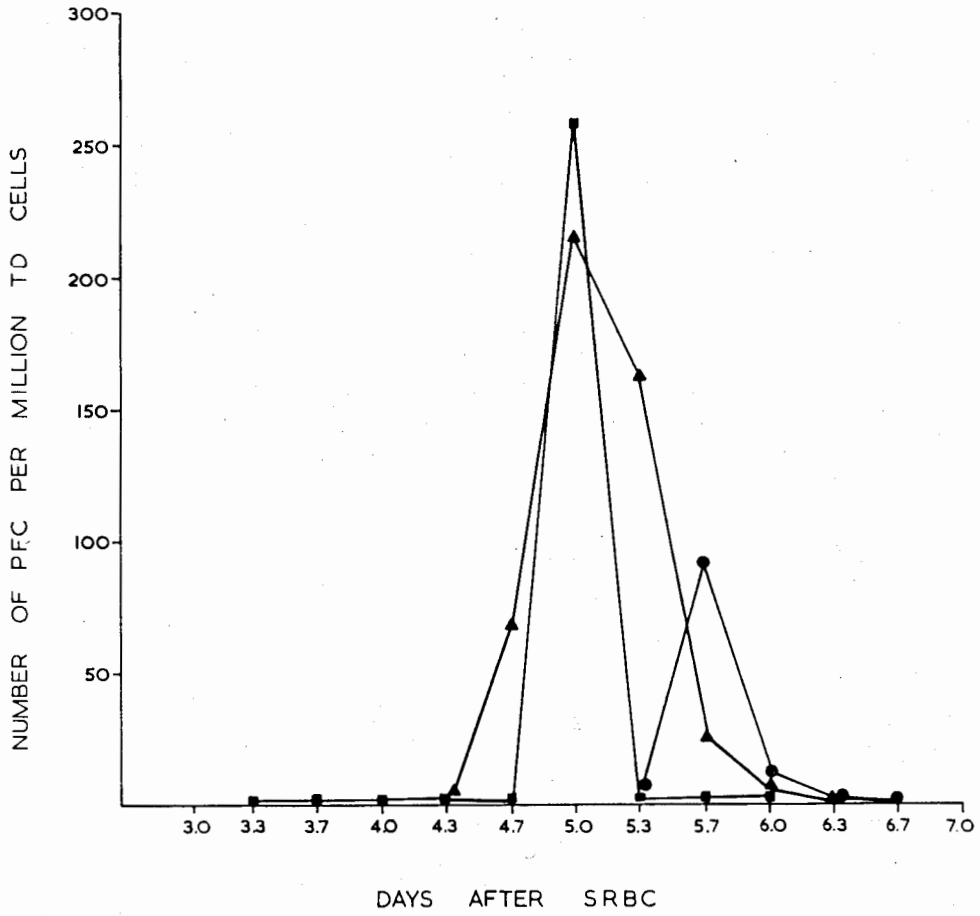


Figure 7. Eight-hourly output of plaque-forming cells (PFC) from the thoracic duct (TD) of normal CBA mice injected intraperitoneally with sheep erythrocytes (SRBC) and cannulated 3 (■), 4 (▲) and 5 (●) days later. Each point represents the value obtained from a pooled sample of lymph collected from 1 to 3 mice.

(ii) Output of cells from the thoracic duct in neonatally-thymectomized mice. CBA mice which had been thymectomized within 24 hours of birth had their thoracic duct cannulated at 5 to 10 weeks of age. In this laboratory, some neonatally-thymectomized mice show signs of wasting disease at 6 to 7 weeks of age. In one experiment, losses from wasting disease were recorded in a group of 14 neonatally-thymectomized CBA mice. The first death occurred at 7 weeks of age, 9 were dead by 10 weeks, and all had died by 12 weeks. Only mice with comparable body weights to those of sham-operated litter mates were used in the output studies. In preliminary experiments, output determinations were performed on a total of 12 thymectomized and 13 5 weeks old sham-operated control mice. Over a 24 hour drainage period an average of 0.96 million cells (with a standard error of the mean of 0.16) was drained from thymectomized mice in contrast to 41.3 ± 5.8 million from the controls ($P < 0.001$).

A more detailed study using 5 to 6 weeks old CBA mice was then undertaken. Thoracic duct cannulations were performed in 4 neonatally-thymectomized mice (mean body weight of 15.8 ± 1.1 gm) and 4 sham neonatally-thymectomized controls (mean body weight of 16.5 ± 1.1 gm) and the output of lymph and cells measured every 3 hours for 48 hours. The results are shown in Figure 8, and the differential counts of cells collected between 0 to 6 and 24 to 36 hours are presented in Table 1, groups 2 and 3. It can be seen in the figure that the output of cells from neonatally-thymectomized mice in each 3 hour collection period remained well below 1 million whereas that from control mice varied from 1.5 to 17 million. In 48 hours, 86.0 ± 19.5 million cells were drained from nonthymectomized mice. The thymectomized mice, on the other hand, produced 2.65 ± 0.15 million cells, this reduced output being significantly different from that in the control group ($P < 0.02$). From the table it is evident that there is a further reduction in the number of small lymphocytes in the thoracic duct of neonatally-thymectomized mice. In fact, the output of small lymphocytes

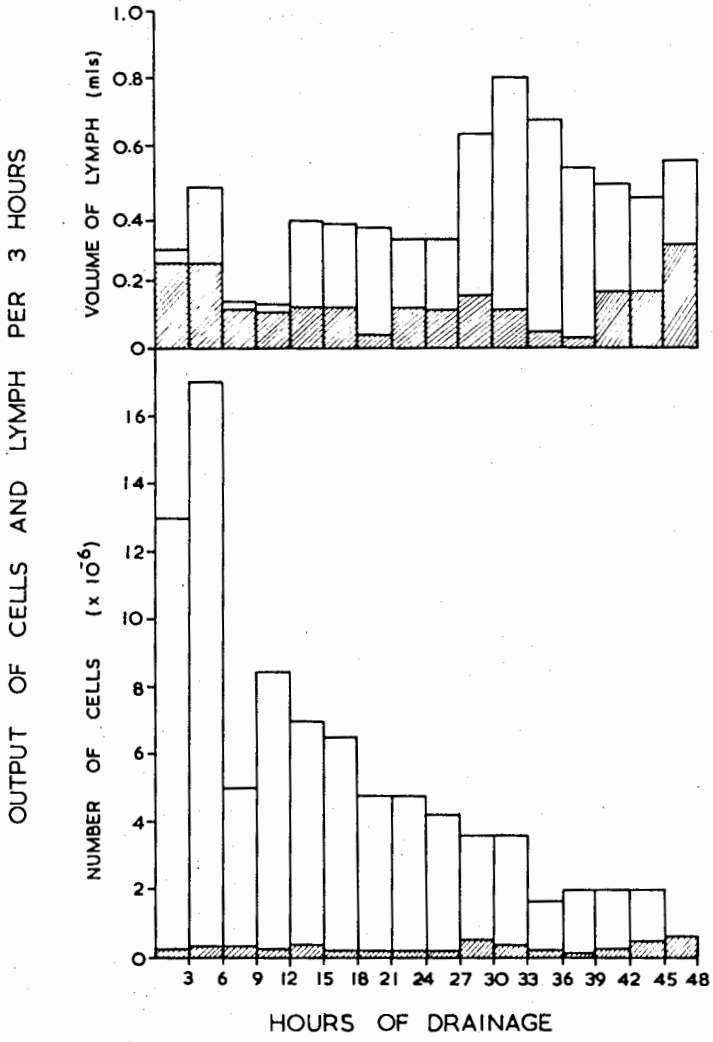


Figure 8. Three-hourly output of cells and lymph from the thoracic duct of 5 to 6 weeks old CBA mice, sham-thymectomized (unshaded blocks) or thymectomized (shaded blocks) within 24 hours of birth. Each block in the histograms represents the average output from 4 mice.

can be calculated to be approximately 2% of that from intact mice of similar age and body weight. This paucity of lymphocytes is reflected in the peripheral blood. The average total peripheral blood leucocyte count was lowered by 77% from 13,000 $\pm$ 2,500 in 6 sham-thymectomized mice to 3,000 $\pm$ 500 (12 mice) in the neonatally-thymectomized group. Neutrophils were reduced in number by about 50% and lymphocytes by almost 90%.

The 3-hourly output of lymph fluctuated and in general could not be correlated with the output of cells. The average total volume of lymph drained was 2.23 $\pm$ 0.16 ml in the thymectomized group and 6.84 $\pm$ 3.4 ml in the sham-operated controls, this difference being not significant.

(iii) Output of cells from the thoracic duct in adult-thymectomized mice. Male CBA mice were thymectomized or sham thymectomized at 7 weeks of age and at various times after the operation, 6 to 12 mice were taken from each group for thoracic duct cannulation. The total numbers of cells and volumes of lymph drained in 48 hours at 1, 6, 10, 36, 52 and 95 weeks after thymectomy or sham thymectomy are shown in Figure 9. Considerable difficulty was encountered in maintaining a functional fistula in older mice and many were discarded because of the appearance of blood in the cannula or because of the cessation of lymph flow for prolonged periods of time. The average number of cells drained from thymectomized mice fell from 107.7 million one week after thymectomy to 18.9 million 94 weeks later. The output from intact mice also decreased over this 2 year period from 103.9 million to 43.4 million. The mean output of cells from thymectomized mice was less than that from the control mice at all time points other than 1 week after thymectomy. This difference was statistically significant only at 52 weeks ($P < 0.01$) and 95 weeks ($P < 0.02$). A significant difference in the output of lymph from the 2 groups was not apparent at any one of the time points.

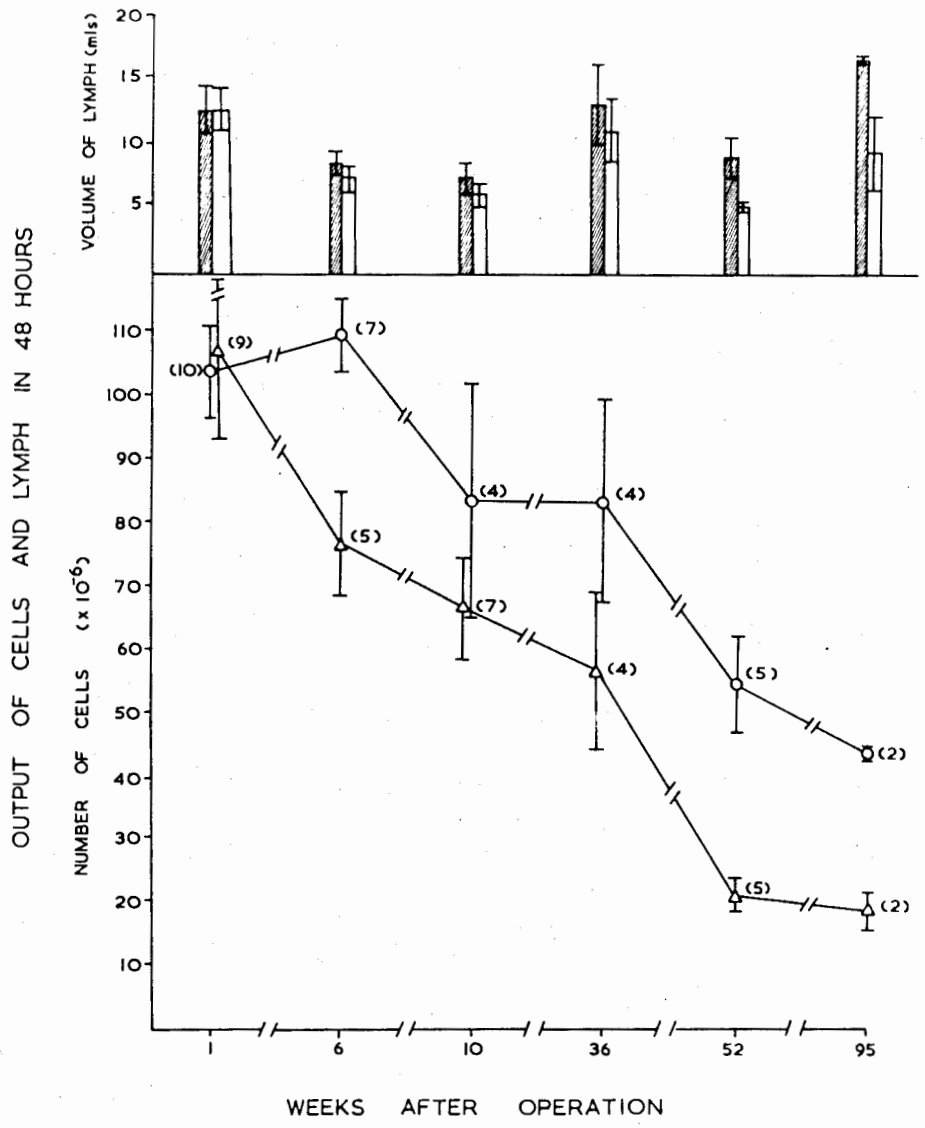


Figure 9. Total 48-hour output of cells and lymph from the thoracic duct of CBA mice thymectomized (Δ and unshaded blocks) or sham thymectomized (O and shaded blocks) at 7 weeks of age and subjected to thoracic duct cannulation at various times thereafter. The number of mice contributing to each point is shown in brackets and the limits of two standard errors of each mean are indicated by the vertical bars.

Differential counts of thoracic duct cells were performed at 6, 36 and 95 weeks after operation and the results are shown in Table 1, groups 4 and 5. It is apparent that only at a time when the total number of cells from thymectomized mice was significantly reduced was the proportion of small lymphocytes noticeably diminished. It can be calculated from Figure 9 and the table that the output of thoracic duct small lymphocytes from 2 year old adult-thymectomized mice was approximately 36% of that from intact mice of the same age.

In an attempt to detect any physiological changes in the population of thoracic duct cells following adult thymectomy, groups of 36 weeks old adult-thymectomized and sham adult-thymectomized mice received 5 intraperitoneal injections of tritiated thymidine (2.5 μc/gm body weight per injection of Thymidine -6-T(n), Batch 26, The Radiochemical Centre, Amersham, England, specific activity 18, 100mc/mM) at 12-hourly intervals. Immediately after the last thymidine injection the mice were cannulated and smears prepared from the first 12 hours collection of cells. The slides were processed autoradiographically by Miss M. Dorr using Kodak NTB/2 dipping emulsion, according to the technique routinely used in these laboratories and described by Prescott, 1964. Representative slides were developed after an exposure time of 4 weeks and labelled cells enumerated. Sections of spleen, mesenteric lymph node and Peyer's patch were processed and developed in parallel.

Heavily-labelled cells were present in large numbers in the red pulp of the spleen and were abundant in the white pulp of the spleen and in the lymph nodes and Peyer's patches. Virtually all epithelial cells of the gut mucosa had numerous grains over the nuclei and grains were scattered diffusely over germinal centre cells in the lymph nodes and Peyer's patches. No lightly-labelled cells were detected in counts of 2,000 cells in smears of thoracic duct cells from either adult-thymectomized or sham-operated mice. Thoracic duct cell smears from 2 adult-thymectomized mice contained an average of 4.6% heavily-

labelled cells and those from 2 sham-thymectomized mice contained an average of 3.4%. It is apparent that very little difference in the pattern of in vivo tritiated thymidine incorporation could be detected in thoracic duct cells from mice thymectomized or sham operated as adults, 29 weeks previously. The results of this single experiment do not support the contention that the output of cells in adult-thymectomized mice is maintained, for some months after the operation, by an increase in the number of short-lived cells or cells newly formed within lymphoid organs.

(iv) Output of cells from the thoracic duct in heavily-irradiated mice. Thoracic duct output studies were next performed in irradiated CBA male mice which had been thymectomized or sham thymectomized at 6 weeks of age. The mice received 900 rads whole body x-irradiation at 8 weeks of age and were injected intravenously, on the day of irradiation, with 10 million syngeneic bone marrow cells from 8 weeks old donors. Groups of mice had their thoracic duct cannulated at either 2 days or 2, 3, 4 or 8 weeks after irradiation and bone marrow protection. The 48 hour cell and lymph outputs are shown in Figure 10. The output of cells from intact mice had fallen from an expected 100 million (Figure 9) to 5 million 2 days after irradiation. At two weeks the 48 hour output had not changed but, over the ensuing 6 weeks, the size of the pool increased progressively to approximately 60 million cells. By contrast, no significant increase in the size of the pool was recorded from 2 to 8 weeks after irradiation and marrow protection in adult-thymectomized mice. The number of cells recovered at 4 and 8 weeks from such mice was significantly less than the number harvested from nonthymectomized mice ($P < 0.01$ and $P < 0.05$, respectively). No significant difference in the output of lymph from the 2 host types was detected at any one time.

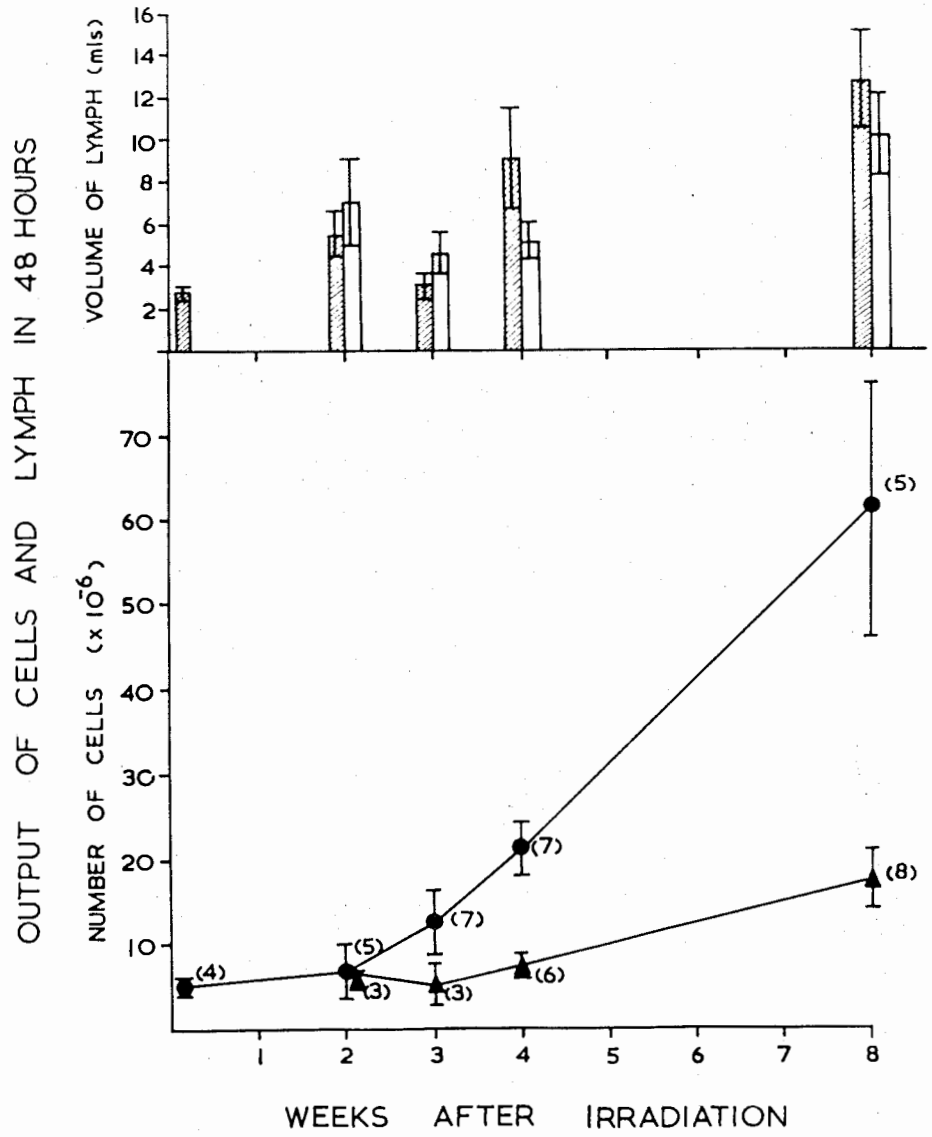


Figure 10. Total 48 hour output of cells and lymph from the thoracic duct of adult thymectomized (▲ and unshaded blocks) and sham adult thymectomized (● and shaded blocks) CBA mice, heavily irradiated at 8 weeks of age and protected with 10 million syngeneic bone marrow cells. Groups of mice had their thoracic duct cannulated at 2 days to 8 weeks after irradiation and marrow injection. The number of mice contributing to each point is shown in brackets and the limits of two standard errors of each mean are indicated by the vertical bars.

Differential counts of thoracic duct cells (Table 1, groups 6 and 7) indicate that the percentage of small lymphocytes in non-thymectomized mice had not, at 8 weeks, reached the expected pre-irradiation level of > 90%. The difference between the two groups, in the proportion of small lymphocytes, was impressive only at 4 weeks after irradiation. The high number of smudges in the smears of cells from thymectomized mice may have accentuated this difference. By 8 weeks, smears from both groups contained in excess of 80% small lymphocytes.

(v) Effect of multiple injections of thymus cells on the thoracic duct cell output in neonatally-thymectomized mice. Neonatally-thymectomized CBA mice were injected intraperitoneally with 110 to 200 million thymus cells from 1 to 10 day old CBA/T6T6 donors in 5 or 6 doses of 10 to 40 million cells. At 6 weeks of age the mice had their thoracic duct cannulated, the output of cells measured over a 48 hour drainage period, and the cells injected into irradiated (800 rads) 5 weeks old (CBA x C57BL)F_1 hybrid recipients. The spleens and mesenteric lymph nodes of these irradiated mice were removed 4 days later and chromosome cytological analyses performed. Twenty eight of 31 suitably-spread mitotic figures carried the marker chromosomes of the original thymus donor type. The mean output of cells from 4 mice which drained for 48 hours was 23.8 ± 2.9 million and the volume of lymph drained was 6.45 ± 0.73 mls. Both these values differ significantly ($P < 0.01$) from the 2.65 million cells and 2.23 ml lymph drained from uninjected neonatally-thymectomized CBA mice.

In another experiment, designed simply as a control of the cytological analyses, neonatally-thymectomized CBA/T6T6 mice were injected intraperitoneally with 350 million thymus cells from 1 to 10 day old CBA mice in 7 doses of 10 to 150 million cells. One mouse was subjected to thoracic duct cannulation at 6 weeks of age. The 12 million thoracic duct cells, emerging in the first 16 hours of drainage,

were injected into a single irradiated F_1 mouse as in the experiment described above. The spleen and mesenteric lymph node were removed 4 days after injection and cell smears prepared for chromosome identification. Of the 21 mitotic figures scored, only 3 carried the T6T6 marker chromosomes. Since no mitotic figures were seen in uninjected irradiated (CBA x C57BL)F_1 hybrid mice, the 18 nonT6T6 cells were most probably CBA cells and thus of the thymus donor type.

The finding that the majority of thoracic duct cells stimulated into division were of thymus donor type, does not give any indication of the proportion of thymus donor type cells in the thoracic duct cell population. The availability of specific anti-C57BL and anti-CBA sera led to an attempt to increase the thoracic duct cell output in neonatally-thymectomized CBA mice with (CBA x C57BL)F_1 thymus cells. It was thought that, by using cytotoxicity tests or fluorescent antibody techniques, the proportion of thymus or thymus-derived cells in the thoracic duct lymphocyte population could be measured directly. Accordingly, 7 neonatally-thymectomized CBA mice were injected with 340 million (CBA x C57BL)F_1 thymus cells from 1 to 7 day old donors in a total of 12 injections. The intra-peritoneal injections were commenced on the second day of life and were continued for 14 weeks at which time thoracic duct fistulae were established. Surprisingly, the output of lymphocytes was not increased over that from uninjected 5 weeks old neonatally-thymectomized CBA mice. The mice did not show any signs of wasting disease but, at the time of cannulation, the spleens were noticed to be grossly enlarged. Two unoperated mice were then grafted with C57BL and Balb/c skin. The Balb/c skin was rejected within 17 days without any evidence of hair growth but the C57BL skin grafts are still intact, with luxuriant growths of hair, 4 months after grafting. On the basis of these findings it seems that the mice were not sensitized against C57BL histocompatibility antigens and were thus unlikely to have eliminated the injected (CBA x C57BL)F_1 hybrid thymus cells.

The enlarged spleens, on histological examination, exhibited signs of gross myeloid hyperplasia. The implications of these findings will be discussed in Section III.

(2) Investigations into the immunological defects in thymectomized mice

Thoracic duct lymphocytes have been implicated in certain immunological responses (Section IB). Hence, the reduced number of cells in the thoracic duct lymph of neonatally-thymectomized mice may contribute to the inability of such mice to respond in normal fashion to a variety of antigenic stimuli (Section IC). Before embarking on studies to compare the immunological performance of thoracic duct cells from normal and neonatally-thymectomized mice, it was first necessary to determine the immunological status of neonatally-thymectomized mice from the CBA colony maintained at the Hall Institute.

(i) Immunological reactivity of neonatally-thymectomized CBA mice. Mice which had been thymectomized or sham thymectomized within 24 hours of birth were made available to Dr. N.S. Weiss who injected them intraperitoneally at various ages with sheep erythrocytes (SRBC). The data on the peak number of PFC produced per 10^6 nucleated spleen cells and per spleen have been published (Weiss, Mitchell and Miller, 1967). At 10 days of age the neonatally-thymectomized mice failed to respond to SRBC by producing PFC in excess of the number in the spleens of unimmunized mice but control mice were already able to produce from 10^3 to 10^4 PFC per spleen.

At 3 weeks of age about 10^3 PFC were detected in the spleens of thymectomized mice and numbers exceeding 10^4 were present in the spleens of controls. From 3 to 8 weeks of age, the mean peak number of PFC in the spleens of thymectomized mice did not change and was $> 1 \log_{10}$ lower than that in sham-thymectomized mice. Neonatally-thymectomized CBA mice thus exhibited both a delay in the development of SRBC reactivity and a splenic PFC response which was, at all ages, lower than/in non-thymectomized mice.

Defects in homograft immunity following neonatal thymectomy have been well documented. The capacity of the neonatally-thymectomized CBA mice in this laboratory to reject foreign skin grafts was tested by grafting 7 of these mice at 4 weeks of age and 3 at 10 days of age with C57BL skin. This particular donor strain was chosen since CBA and C57BL mice differ at the strong H_2 histocompatibility locus. All mice died of wasting disease between 6.5 and 10 weeks of age with the grafts intact. By contrast, six normal mice grafted with C57BL skin at 4 weeks of age rejected the grafts within 14 days (mean survival time of 11 days).

(ii) Immunological competence of thoracic duct cells from neonatally-thymectomized mice. Two assay systems were chosen to test the immunocompetence of cells in the thoracic duct lymph of thymectomized and normal mice. The graft-versus-host reaction was employed as a means of assessing the overall competence of lymphocyte populations. In addition, the haemolytic focus assay was used to compare populations of cells for their content of antigen-reactive cells (ARC).

(a) Graft-versus-host activity of thoracic duct cells. Thoracic duct cells were collected from 5 to 6 weeks old neonatally-thymectomized and sham-thymectomized CBA mice. Because of the markedly reduced number of cells in the thoracic duct of neonatally-thymectomized mice, many more of these mice were cannulated compared with the number of nonthymectomized cell donors. From 0.1 to 2 million cells in 0.05 ml PBS were injected intravenously into (CBA x C57BL)F_1 hybrid mice within 24 hours of birth. Some of the F_1 newborns were injected with PBS and appropriate numbers of F_1 thoracic duct cells. Such control mice were always included in the same litter as recipients of parental thoracic duct cells.

The effect of the inoculated cells was determined 9 days after injection by measuring the degree of splenomegaly in relation to the total

body weight. The splenic index is the spleen : body weight ratio in F_1 recipients of parental cells divided by the spleen : body weight ratio in control mice. An index of 1.5 or more is considered evidence of a graft-versus-host reaction. It can be seen in Figure 11 that thoracic duct cells from sham-thymectomized mice, in numbers >0.5 million, uniformly produced such a reaction. By contrast, inocula of 0.4 to 1.0 million thoracic duct cells from neonatally-thymectomized CBA mice failed to induce significant splenomegaly in newborn F_1 recipients.

(b) Antigen-reactive cells in thoracic duct lymph. As mentioned previously (Section IB), the number of ARC in a population of cells can be determined by injecting various numbers of the cells, together with SRBC, into irradiated mice and enumerating the haemolytic foci appearing in the spleens 8 days later. The haemolytic focus-producing capacity of spleen and thoracic duct cells from sham neonatally-thymectomized mice was first determined. One half to 5 million spleen cells or 0.25 to 1.2 million thoracic duct cells were injected together with SRBC into 8 to 10 weeks old syngeneic recipients which had received 900 rads total body x-irradiation not more than 6 hours previously. Other heavily-irradiated mice were injected with SRBC only, lymphoid cells only, or were left uninjected. The spleens were sectioned 8 days later and the "active areas" or haemolytic foci enumerated. The results are presented in Figure 12. A linear relationship exists between the number of nucleated spleen or thoracic duct cells injected and the number of foci appearing in the spleen. The average number of foci in the spleens of uninjected irradiated mice or in those receiving cells or SRBC was < 1. By extrapolating to the ordinate, the lines of best fit obtained with SRBC and various numbers of spleen and thoracic duct cells, the point of intersection was found to be not significantly different from one haemolytic focus. From these results, and also those from other laboratories (Section IB), the "background" number of foci in these and subsequent experiments was considered to be one and was subtracted from

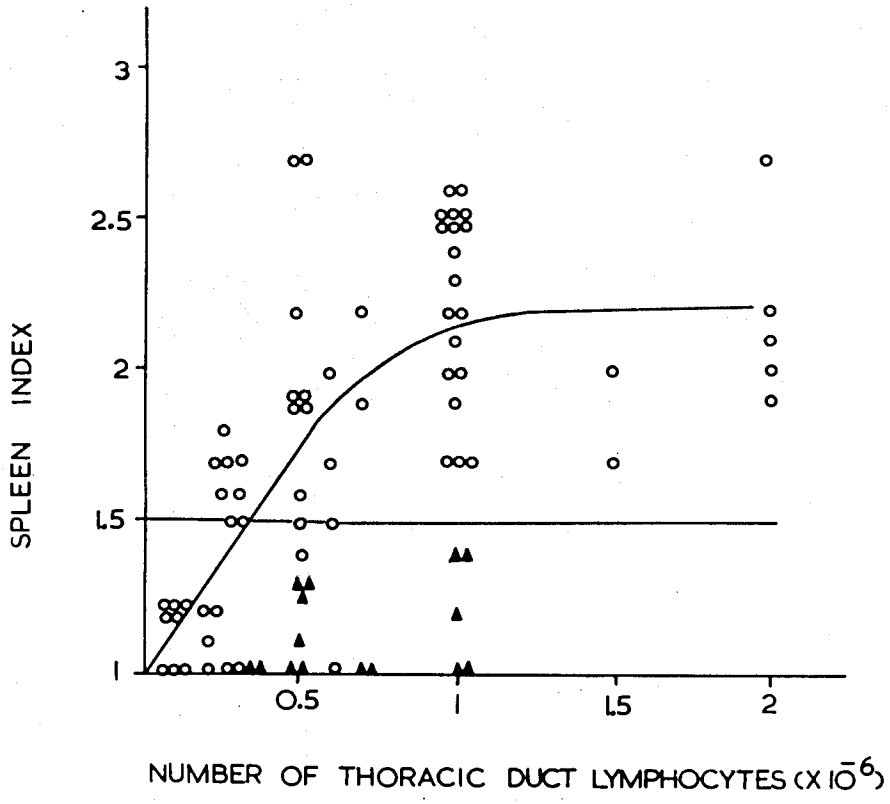


Figure 11. Splenic indices of 9 day old (CBA x C57BL)F_1 hybrid mice injected at birth with various numbers of thoracic duct cells from 5 to 6 weeks old neonatally thymectomized (▲) and sham neonatally thymectomized (O) CBA mice. A spleen index of $\geqslant 1.5$ is considered evidence of a graft-versus-host reaction and each point represents the value obtained from a single F_1 recipient.

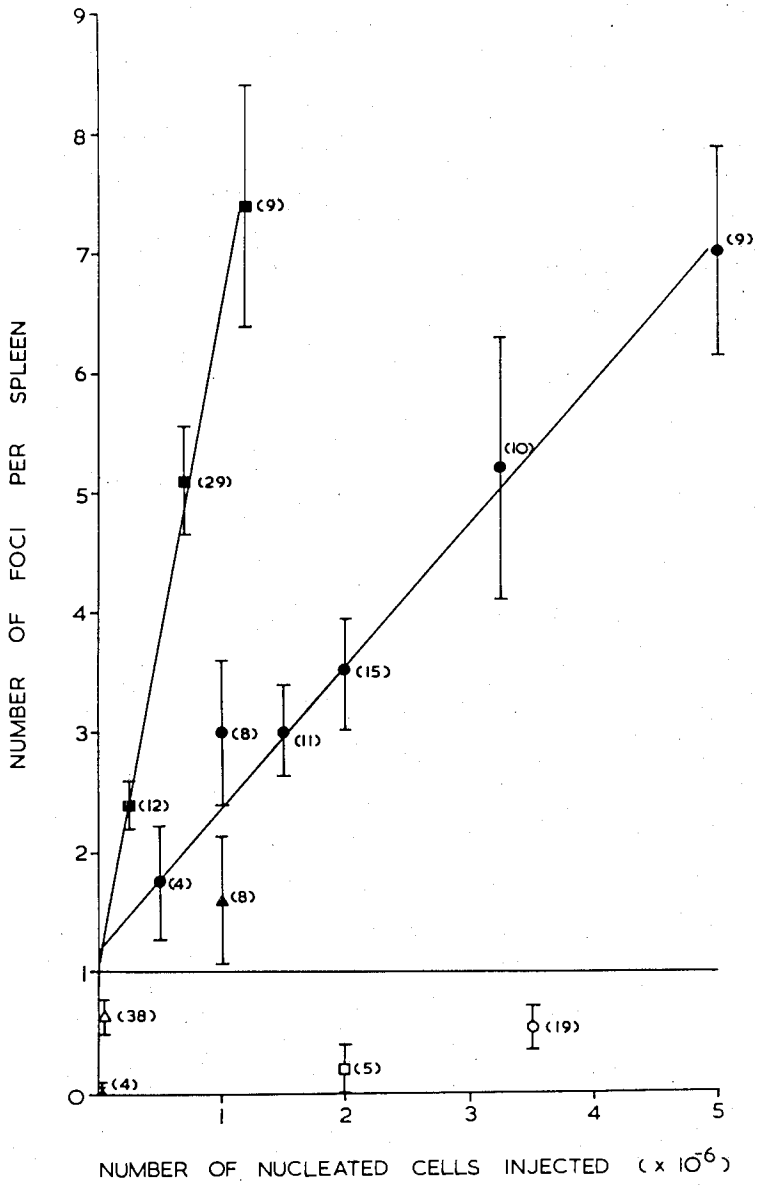


Figure 12. Mean number of haemolytic foci appearing in the spleens of CBA mice 8 days after irradiation and injection of sheep erythrocytes (SRBC) and/or syngeneic cells from various sources: (●) spleen cells from intact mice + SRBC; (■) thoracic duct cells from intact mice + SRBC; (▲) thoracic duct cells from neonatally thymectomized mice + SRBC; (○) 1 to 6 million spleen cells only; (□) 1 to 3 million thoracic duct cells only; (Δ) SRBC only; (X) no injection. The number of mice contributing to each point is shown in brackets and the limits of two standard errors of the mean are represented by the vertical bars.

all the experimental results.

It is apparent from Figure 12 that the slope of the line for thoracic duct cells is steeper than that for spleen cells. There is thus a higher proportion of ARC in the thoracic duct lymph. Thoracic duct cells from 5 to 6 weeks old neonatally-thymectomized donors were next tested for their content of ARC. The mean number of foci following an injection of one million cells was 0.6 and this number contrasts with approximately 6 following an injection of an equivalent number of thoracic duct cells from nonthymectomized mice.

By using a double transplantation technique in which spleens from inoculated irradiated mice were transferred after 2 hours into further irradiated mice, Kennedy et al. (1965a) and other workers have shown that approximately 10% of the total ARC in the inoculum localize in the spleen (Section IB). This figure cannot be expected to be correct since it does not take into account further recruitment of ARC after 2 hours and hence the total number of ARC in a particular population of cells cannot be determined accurately. There seems to be no reason, however, for not using this figure when comparing two populations of cells for their content of ARC. The reported figure of the fraction of ARC localizing in the spleen of irradiated mice was confirmed during the present investigations. Each irradiated mouse in a group of 10 mice (A) was injected with 2×10^6 spleen cells together with SRBC. In another group (B), each irradiated recipient was injected with 20×10^6 spleen cells. Group B mice were killed at 2 hours and spleen cells (20 million) transferred into further irradiated mice (group B′) together with SRBC. The average number of foci appearing in the spleens 8 days later in group A was divided by the average number in the spleens of group B′ multiplied by 10. This ratio, expressed as a percentage, was 7%. Furthermore, in a small experiment involving only 4 group B′ mice, the fraction of ARC in thoracic duct cell inocula localizing in the spleen was found to have a mean value of slightly less than 10%. In all subsequent calculations the localization percentage was considered

to be 10 for both spleen and thoracic duct cell inocula.

Figure 12 shows that 1 million thoracic duct cells from non-thymectomized mice produced, on the average, 6 haemolytic foci. Assuming that 10% of the ARC lodge in the spleen and that the 48 hour thoracic duct lymphocyte pool in mice of this age is approximately 80 million cells (Figure 8), the total number of ARC in the pool is 4,800. By applying these same considerations to the thoracic duct cells from neonatally-thymectomized mice, the number of ARC per million cells is 6. With a pool size of 3 million cells, the total number of ARC is less than 20. The thoracic duct cell population following neonatal thymectomy is thus deficient, not only in the number of cells present, but also in the relative and absolute number of antigen-reactive cells.

Spleen cell inocula produced approximately 1.2 foci per million injected cells in response to sheep erythrocytes in irradiated mice (Figure 12). The spleens of 5-6 weeks old sham-thymectomized mice contain in excess of 10^8 nucleated cells. The total number of ARC per spleen is therefore circa 1,200. As mentioned, this figure may be misleading and could conceivably be grossly inaccurate. For comparative purposes, however, the figure is useful and will be employed in studies to be described in the following section.

(iii) Antigen-reactive cell precursors in bone marrow and thymus

Since the number of thoracic duct ARC was markedly reduced following neonatal thymectomy, the thymus may be involved in the generation of these cells from some precursor cell population. The regeneration of the pool of thoracic duct lymphocytes in irradiated, marrow-protected CBA mice was dependent upon the presence of the thymus (Figure 10). Furthermore, the restoration of PFC reactivity to SRBC in such mice was found to be influenced by the presence of the thymus. In 4 separate experiments, 8 to 24 weeks old CBA male mice received 900 rads total body x-irradiation and 10 million syngeneic bone marrow cells 1 to 3 hours after irradiation. Some of the mice had been thymectomized at

6 to 7 weeks of age. At various times between 2 and 10 weeks after irradiation, groups of mice were injected intravenously with SRBC and the number of PFC in the spleens determined 4 to 5 days later. The results of the 4 experiments were similar and are combined in Figure 13. It can be seen that the number of PFC appearing in the spleens of both thymectomized and nonthymectomized irradiated and marrow protected mice was < 500 at 2 weeks after irradiation. By 4 weeks the number in the nonthymectomized group had reached 20,305 while the number in the thymectomized group was still < 2,000. The 4 - 5 day PFC response to SRBC at 6 to 10 weeks had returned to levels comparable with those in normal mice of similar age (Martin and Miller, 1968) only in the group of mice in which the thymus was present. The thymectomized mice could produce, on the average, only about 3,000 PFC in the spleen at these time points.

The results of a time course study of thymus regeneration after irradiation are shown in Figure 14. Normal mice received 900 rads x-irradiation at 7 to 8 weeks of age and were injected with 10 million syngeneic bone marrow cells immediately after irradiation. The thymus weight fell from approximately 40 mgm at the time of irradiation to 10 mgm 2 days later. No increase was apparent until after 1 week and preirradiation thymus weights were attained 3 to 4 weeks after irradiation. Studies on the population of ARC in the spleens of mice recovering from irradiation were then undertaken.

Eight to 12 weeks old CBA male mice received 900 rads x-irradiation and injections of 10^3, 10^5, 10^6 or 10^7 syngeneic bone marrow cells within 4 hours of irradiation. Some mice had been thymectomized at 6 weeks of age and others received bone marrow from neonatally-thymectomized donors. At 1 to 10 weeks after irradiation, bone marrow-incubating hosts were killed, the spleens removed, and single cell suspensions prepared. Various numbers of cells from the spleen cell suspensions were injected together with

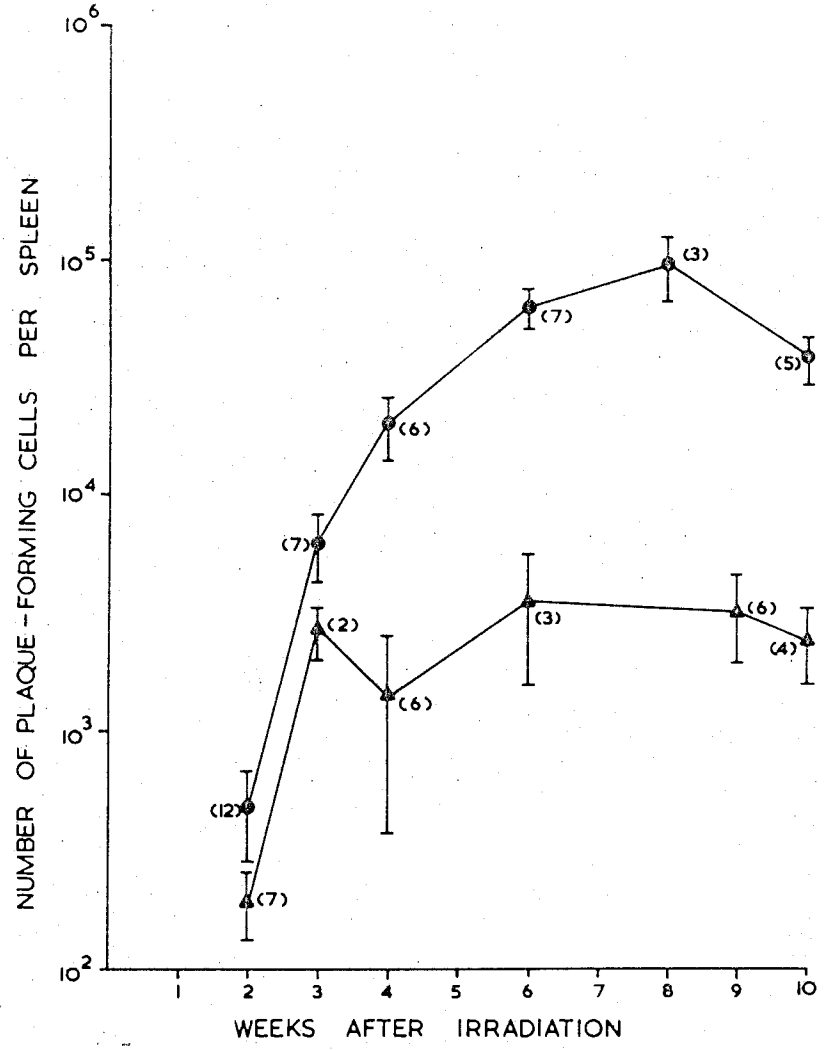


Figure 13. Appearance of plaque-forming cells in the spleens of sham adult thymectomized (●——●) and adult thymectomized (▲——▲) CBA mice at various times after receiving 900 rads x-irradiation and 10 million syngeneic bone marrow cells. Splenic plaque-forming cells were enumerated 4 to 5 days after the challenge injection of sheep erythrocytes. The number of mice contributing to each point is shown in brackets and the limits of two standard errors of each mean are indicated by the vertical bars (semi-log scale).

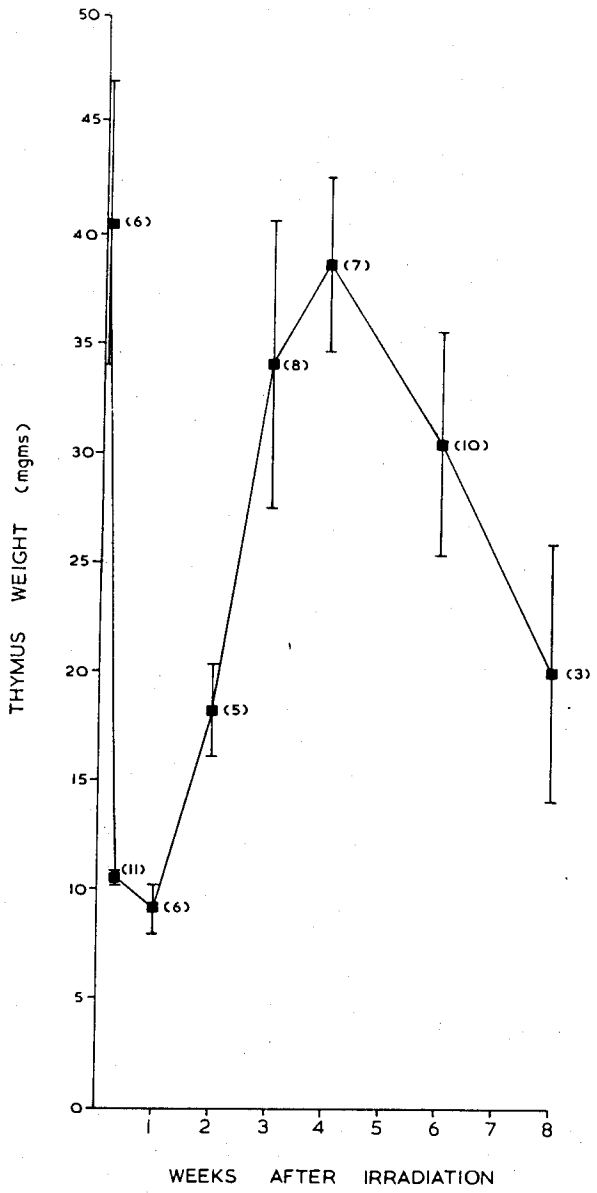


Figure 14. Weight of the thymus in CBA mice at various times after 900 rads total body x-irradiation and injection of 10 million syngeneic bone marrow cells. Each point represents the mean value obtained from the number of mice shown in brackets and the vertical bars indicate the limits of two standard errors of the mean.

SRBC into further irradiated mice. Eight days later the spleens of the secondary hosts were removed and the number of haemolytic foci determined. From a knowledge of the average spleen cell number in the primary hosts, the number of cells injected into the secondary hosts, and the number of foci produced, the average total number of ARC in the spleens of the primary hosts can be calculated.

The essential features of the experimental design are shown in Figure 15 and the numbers of ARC in the spleens of nonthymectomized irradiated mice incubating bone marrow cells from either normal or neonatally-thymectomized mice are shown in Tables 2 and 4 respectively. ARC in the spleens of adult thymectomized, irradiated and marrow-inoculated mice are indicated in Table 3 and all the results are summarized in Figure 16. This figure shows that no ARC could be detected in any of the spleens at 1 week after irradiation. At 2 weeks, however, ARC had appeared in the spleens of nonthymectomized hosts incubating 10^6 to 10^7 normal bone marrow cells or bone marrow cells from neonatally-thymectomized mice. By 3, 4 and 5 weeks, ARC could be found in numbers approximating the 1,000 or so expected to be present in the spleen of normal mice. The situation in thymectomized hosts was strikingly different. Even after 10 weeks of bone marrow incubation the number of ARC in the spleen had not increased above 200. The number of ARC in the spleen of one surviving nonthymectomized irradiated mouse, inoculated with as few as 10^3 bone marrow cells, had reached normal levels 10 weeks after irradiation (Table 2).

These data indicate that the bone marrow of normal and neonatally thymectomized mice contains precursor cells which differentiate into ARC, only under the influence of the thymus. As expected from the data in Figure 13, no thymus regeneration was evident either macroscopically or microscopically in any of the mice at autopsy until after 1 week of bone marrow incubation. From the graphs in Figures 14 and 16 it is evident that thymus regeneration coincides with the appearance

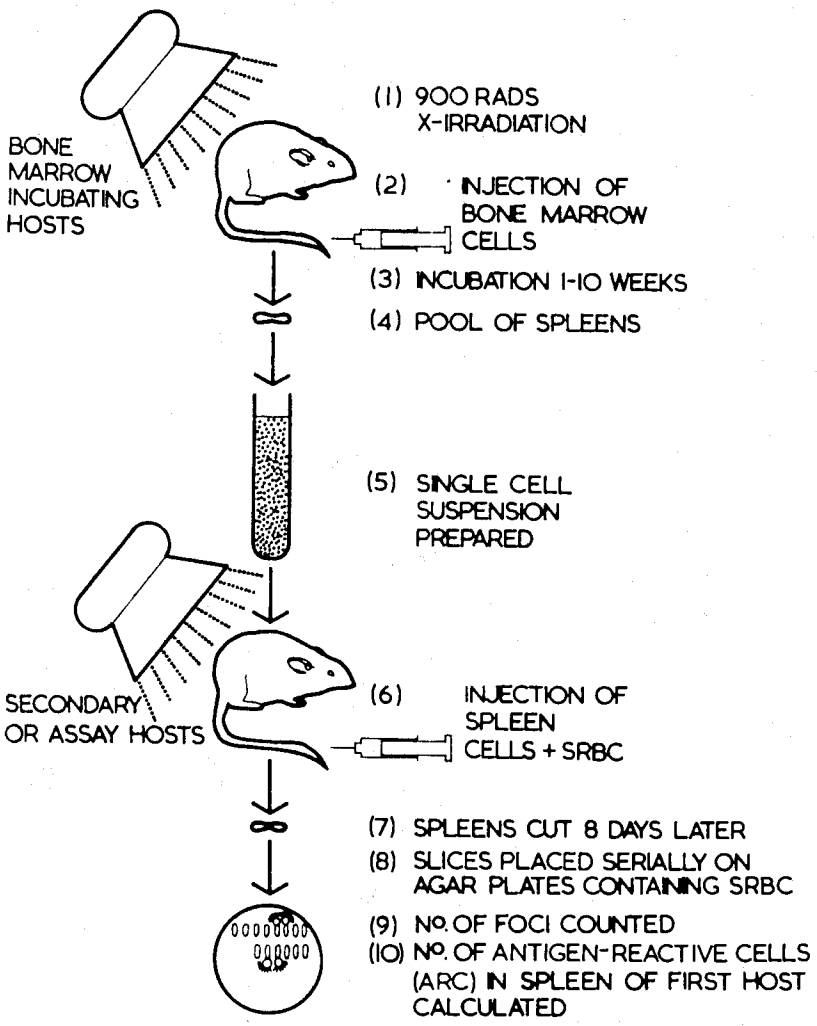


Figure 15. Experimental protocol for the detection of antigen-reactive cells in the spleens of irradiated mice incubating bone marrow cells.

Table 2

Number of Antigen-Reactive Cells (ARC) in the Spleens of Heavily Irradiated CBA Mice Incubating 10^3 to 10^7 Bone Marrow Cells from Normal Syngeneic Donors for 1 to 10 Weeks

Bone marrow incubating hosts				ARC assay hosts	
No. of bone marrow cells injected	Weeks after irradiation	No. of mice	Average No. of cells per spleen ($\times 10^{-6}$)	No. of mice	No. of ARC per spleen
1×10^3	1	5	8	7	0
	2	4	60	8	0
	3	6	160	8	150
	10	1	105	6	1360
1×10^5	1	6	10	6	0
	2	9 (3)⁂	90	12 (3)⁂	50, 200, 240
	3	15 (3)	106	23 (3)	120, 400, 450
1×10^6 to 1×10^7	1	11 (2)	58	10 (2)	0, 0
	2	38 (9)	109	80 (9)	90, 100, 130 144, 300, 300 380, 420, 475
	3	12 (3)	103	24 (3)	270, 1900, 2100
	4	7 (2)	101	12 (2)	360, 1200
	5	5	106	7	1250

⁂ Number in brackets refers to the number of individual experiments.

Table 3

Number of Antigen-Reactive Cells (ARC) in the Spleens of Heavily Irradiated CBA Mice Thymectomized in Adult Life and Incubating 10^6 to 10^7 Bone Marrow Cells from Normal Syngeneic Donors for 1 to 10 Weeks

Bone marrow incubating hosts			ARC assay hosts	
Weeks after irradiation	No. of mice	Average No. of cells per spleen (x 10^{-6})	No. of mice	No. of ARC per spleen
1	5	15	5	0
3	16 (5)⁂	86	36 (5)⁂	7, 14, 23, 36, 53
6	10 (4)	121	28 (4)	50, 110, 145, 210
8	2	76	6	65
9	3	157	8	15
10	1	115	7	130

⁂ Number in brackets refers to the number of individual experiments.

Table 4

Number of Antigen-Reactive Cells (ARC) in the Spleens of Heavily Irradiated CBA Mice Incubating 10^6 to 10^7 Bone Marrow Cells from Neonatally Thymectomized Syngeneic Donors for 1 to 3 Weeks

Bone marrow incubating hosts			ARC assay hosts	
Weeks after irradiation	No. of mice	Average No. of cells per spleen (x 10^{-6})	No. of mice	No. of ARC per spleen
1	6	34	4	0
2	20 (6)⁂	176	40 (6)⁂	73, 100, 120, 250, 400, 600
3	7 (2)	111	9 (2)	520, 1500

⁂ Number in brackets refers to the number of individual experiments.

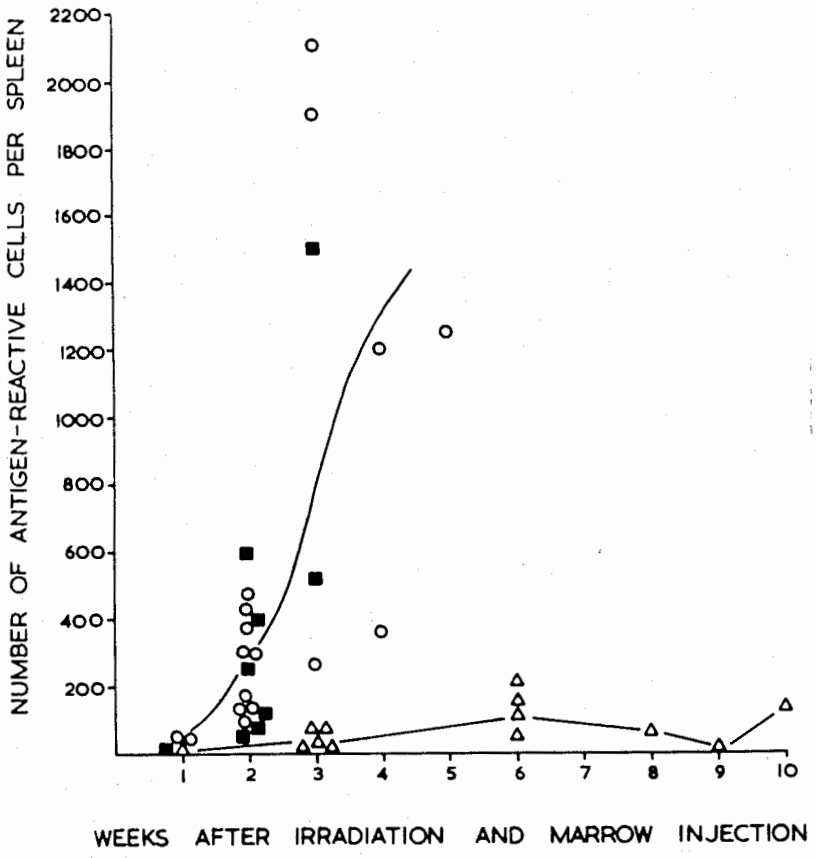


Figure 16. Appearance of antigen-reactive cells in the spleens of adult mice over a 10 week period following 900 rads total body x-irradiation and injection of 10^6 to 10^7 syngeneic bone marrow cells: (O) number in non-thymectomized irradiated mice injected with bone marrow cells from normal donors; (■) number in non-thymectomized irradiated mice injected with bone marrow cells from neonatally thymectomized donors; (Δ) number in adult thymectomized irradiated mice injected with bone marrow cells from normal donors. Each point represents the value obtained in a single experiment (Tables 2,3 and 4).

of ARC in the spleen of nonthymectomized, irradiated and marrow protected mice. The simplest interpretation of this finding is as follows. Bone marrow cells, or bone marrow-derived cells, repopulate the thymus and lymphocytes are exported as ARC or as cells which are destined to mature into ARC within peripheral lymphoid organs. If the hypothesis is correct then thymus cell populations should contain ARC or their immediate precursors, and one might expect that incubating large numbers of thymus cells in irradiated mice would result in an early appearance of ARC in the spleens.

Mice which had received 900 rads x-irradiation at 8 to 12 weeks of age, were injected with 10^8 thymus cells. Since the thymus population lacks haemopoietic stem cells, the mice were destined to die of haemopoietic failure. The spleens from thymus cell-incubating mice were therefore transferred at weekly intervals into successive sets of irradiated hosts. At each passage an aliquot of the pooled spleen cell suspension was injected into other irradiated mice together with SRBC and the number of haemolytic foci determined 8 days later. From Table 5 it is apparent that virtually no ARC were present in the spleens even after 4 passages. In parallel, irradiated mice were injected with 10^8 bone marrow cells and the spleens were transferred at weekly intervals into further irradiated hosts. Aliquots were taken at each transfer and ARC assays performed. Table 5 shows that, in contrast to the situation in which bone marrow cells were allowed to reside for 3 weeks in a single host, the appearance of ARC from bone marrow precursors was largely inhibited by weekly transference of the spleens into sets of irradiated mice. The original inoculum of 10^8 cells must be diluted at each transfer. In this case the number of bone marrow cells incubated for 3 weeks (assuming a 10 fold dilution at each transfer) would be 10^5 cells. Even if this is so, one would expect to find 120 to 450 ARC in the spleen of the final host (Table 2). In none of the passage hosts was thymus regeneration apparent according to both histological and macroscopical criteria.

Table 5

Number of Antigen-Reactive Cells in the Spleens of Heavily Irradiated CBA Mice Incubating, for 1 Week, Spleen Cells Transferred at Weekly Intervals from Successive Sets of Irradiated Mice Initially Injected with Syngeneic Bone Marrow or Thymus Cells

Cells injected into 1st host	Average No. of antigen-reactive cells per spleen			
	2nd transfer host	3rd transfer host	4th transfer host	5th transfer host *
1×10^8 bone marrow cells	NR**	90 (10)⁂	0 (8)	0 (8)
1×10^8 thymus cells	5 (8)	32 (8)	45 (7)	NR

* 2 to 6 hosts were used at each repassage.

** NR = no result owing to death of assay hosts.

⁂ Number in brackets refers to the number of antigen-reactive cell assay hosts.

A fortuitious finding at this stage in these investigations hinted at the existence of a thymus-bone marrow interaction in the production of haemolysins. The experimental design was similar to that of the transfer experiments described above. When thymus cells were stimulated with SRBC in irradiated mice, and the spleens transferred after 1 week to further irradiated mice, the PFC response in the secondary hosts was augmented by the presence of bone marrow cells. The results of a series of experiments employing this basic design are shown in Figure 17 and Table 6. It is evident from Figure 17 that a significant response occurred in the secondary hosts only if 10^8 thymus cells had been incubated with SRBC in the 1st host and if the spleens from these latter hosts were transferred together with bone marrow cells and SRBC. When SRBC in the primary hosts were replaced by horse or rabbit erythrocytes the response to sheep erythrocytes in the secondary hosts was not significantly above that in recipients of spleen cells from primary hosts incubating thymus cells alone (Table 6). Claman et al. (1966a, b) had reported that a mixture of thymus and bone marrow cells was far more effective in producing haemolysins to SRBC in irradiated mice than either cell population alone. The findings of the present study corroborate those of Claman et al. and in addition suggest that thymus cells have first to react with the specific antigen before combination with bone marrow can be expected to give rise to PFC. The results, however, do not provide any information as to which cell type is the immediate precursor of the haemolysin plaque-forming cell: - thymus or bone marrow. The population containing PFC precursors would, by definition (Kennedy et al., 1965a, 1966), contain the ARC.

Table 6

Number of Plaque-Forming Cells Produced in the Spleens of Heavily Irradiated Mice (Secondary Hosts) Injected with Sheep Erythrocytes, Bone Marrow Cells and Spleens from Irradiated Mice Incubating, for 1 Week, Thymus Cells and/or Heterologous Erythrocytes (Primary Hosts)

Group No.	No. of mice	Primary Hosts		Secondary Hosts[A]	
		No. of inoculated thymus cells	Erythrocytes inoculated[*]	Average No. of PFC[**] per spleen($\pm$SE)	P value (cf. group 1)
1	26	1×10^8	SRBC	2,487 $\pm$ 609	-
2	19	1×10^8	-	299 $\pm$ 76	< 0.01
3	10	-	SRBC	200 $\pm$ 105	< 0.01
4	13	1×10^8	HRBC	151 $\pm$ 37	< 0.001
5	7	1×10^8	RRBC	100 $\pm$ 16	< 0.001

A Each secondary host received one spleen equivalent from the pool of cells obtained from the primary hosts together with 10^7 bone marrow cells and sheep erythrocytes.

* 0.1 ml of a 20% erythrocyte suspension. Abbreviations: SRBC = sheep erythrocytes; HRBC = horse erythrocytes; RRBC = rabbit erythrocytes.

** PFC = number of antisheep erythrocyte plaque-forming cells determined 7 days after irradiation.

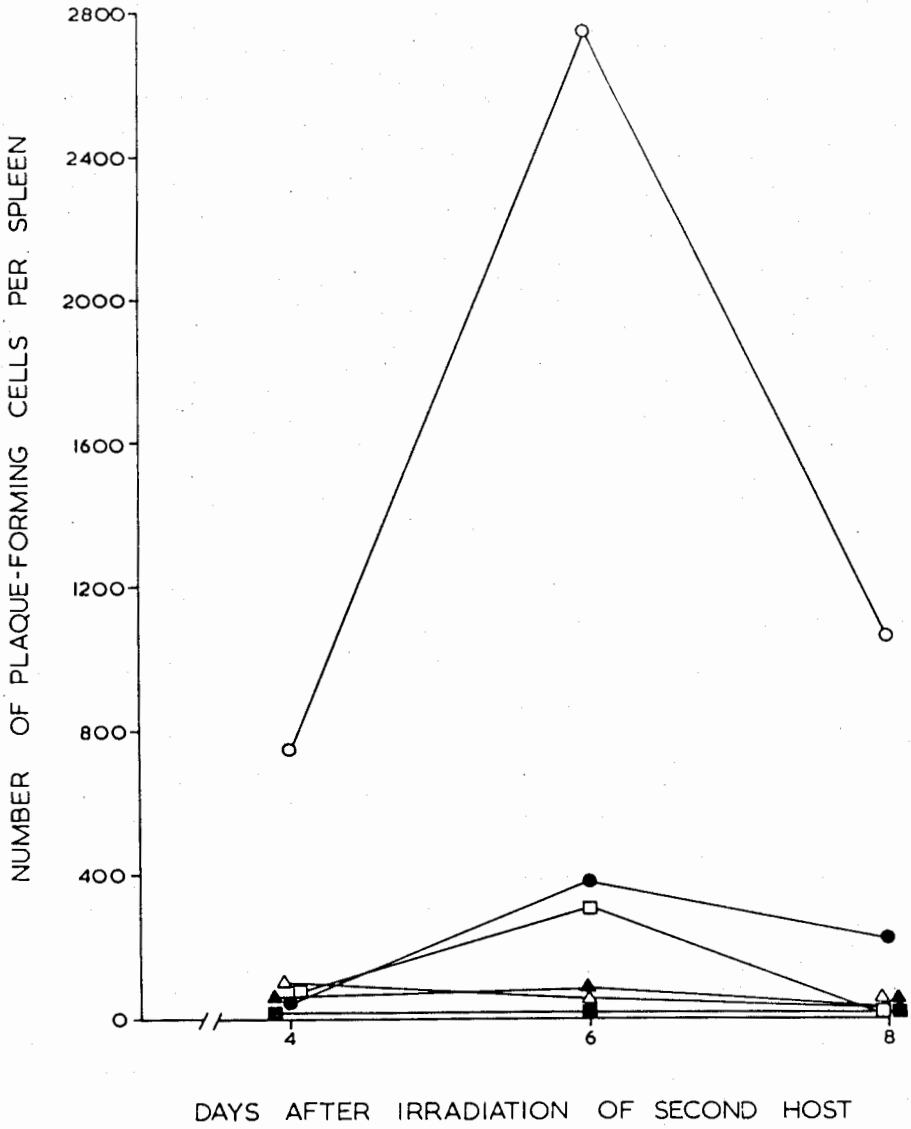


Figure 17. Thymus-marrow interaction in 2 host system. Details in text.

Symbol	Cells injected into first host		Cells injected into second host		
	10^8 T	10^8 SRBC	Spleen from first host	10^7 BM	10^8 SRBC
○	+	+	+	+	+
△	+	+	+	+	-
□	+	+	+	-	+
●	-	+	+	+	+
▲	-	+	+	+	-
■	-	+	+	-	+

Each point represents the value obtained from a single determination made on a pool of spleens from 3 mice. Abbreviations: T = thymus cells; BM = bone marrow cells; SRBC = sheep erythrocytes.

(3) Cell to cell interaction in the immune response to sheep erythrocytes

The evidence for a thymus-bone marrow cell interaction in irradiated mice, together with the finding that the bone marrow of neonatally-thymectomized mice was normal according to one functional criterion, prompted studies into the immunological activity of thymus cell inocula in neonatally-thymectomized mice. If cells present in, or derived from, the thymus and bone marrow are required in the immune response to sheep erythrocytes, then this defective response in neonatally-thymectomized mice should be ameliorated by injections of thymus cells or thymus-derived cells.

(i) Immunological activity of thymus and thoracic duct cells in neonatally-thymectomized mice.

(a) Activity of syngeneic cells. In earlier experiments conducted in collaboration with Dr. N.S. Weiss, (Weiss et al., 1967) the PFC response to SRBC in 10 days old neonatally-thymectomized CBA mice was increased by injections of syngeneic thoracic duct cells in the newborn period. In the present studies 3 to 5 weeks old CBA mice, which had been thymectomized within 36 hours of birth, were injected intravenously with 10 million or 50 million thymus cells or 10 million thoracic duct cells together with SRBC. The donors of the lymphoid cells were 6 to 8 weeks old syngeneic mice. Other thymectomized mice and sham-operated litter mates received SRBC only. The number of PFC appearing in the spleen was determined at 2, 4, 5, 7 and 10 days after injection and the results are presented in Figure 18. Neonatally-thymectomized mice produced on the average a peak number of 2,664 PFC whereas the response in shams reached a peak value of 34,240 PFC (Figure 18,(A)). Injection of thymus and thoracic duct cells increased the response of neonatally-thymectomized mice to levels exceeding 20,000 PFC per spleen (Figure 18, (B) and (C)). The peak response in the group receiving 50 million thymus cells was similar to that in the sham-operated group. The kinetics of PFC production were

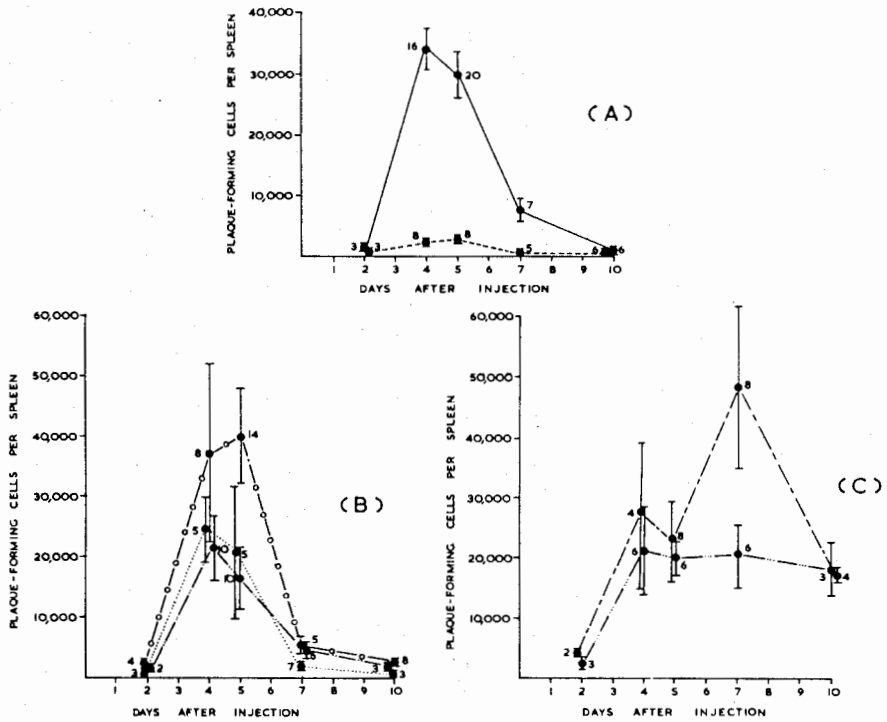


Figure 18. Number of plaque-forming cells in the spleens of 3 to 5 weeks old neonatally thymectomized CBA mice at various times after injection of sheep erythrocytes (SRBC) and thymus or thoracic duct cells.

(A) ●——● sham neonatally thymectomized mice given SRBC only;

●- - - - - - - ● neonatally thymectomized mice given SRBC only.

(B) ●—○—○—● neonatally thymectomized mice given 50 million CBA thymus cells and SRBC;

●—··—··—● neonatally thymectomized mice given 10 million CBA thymus cells and SRBC;

●·····● neonatally thymectomized mice given 10 million (CBA x C57BL)F_1 thymus cells and SRBC

(C) ●—·—·—● neonatally thymectomized mice given 10 million CBA thoracic duct cells and SRBC;

●— — —● neonatally thymectomized mice given 10 million (CBA x C57BL)F_1 thoracic duct cells and SRBC.

The number of mice contributing to each point is indicated and the vertical bars denote the limits of two standard errors of each mean.

similar in recipients of thymus cells and in nonthymectomized mice but the number of PFC in thoracic duct cell-inoculated mice remained elevated from 4 to 10 days after challenge with SRBC (Figure 18, (C)).

The average number of PFC at 4 and 5 days in the spleens of mice receiving 10 million syngeneic thymus and thoracic duct cells was increased significantly over that in thymectomized mice receiving SRBC only (Table 7). By contrast, the PFC response was not increased significantly by an inoculum of 1.5 million thoracic duct cells. The activity of 10 million thymus cells is thus unlikely to be the result of a "contaminant" population of blood-borne lymphocytes in the thymus cell preparation. It can also be seen in Table 7 that the number of "background" PFC in uninoculated neonatally-thymectomized and sham-operated mice was approximately 100 per spleen. Injections of 10 million thoracic duct or thymus cells, in the absence of sheep erythrocytes, resulted in a slight increase in the average number of PFC.

(b) Activity of allogeneic and semiallogeneic cells. Groups of 3 to 5 weeks old neonatally-thymectomized CBA mice were next injected with thymus or thoracic duct cells from allogeneic (C57BL) and semi-allogeneic ((CBA x C57BL)F_1 hybrid) donor mice. The results of a time course study of the appearance of PFC in mice receiving SRBC and 10 million semi-allogeneic thymus or thoracic duct cells are included in Figure 18. As was the case with syngeneic inocula, semiallogeneic cells elevated the PFC response of neonatally-thymectomized mice. Furthermore, the response in mice injected with thoracic duct cells remained elevated when that in thymus-inoculated mice had fallen. A time course study using C57BL thymus or thoracic duct cells was not attempted since the condition of the recipients deteriorated after 5 to 6 days presumably as a result of the effects of a graft-versus-host reaction. The numbers of PFC in the spleens at 4 to 5 days after injection of allogeneic and semiallogeneic

Table 7

Plaque-Forming Cell Response of Neonatally Thymectomized CBA Mice Injected with Sheep Erythrocytes and Syngeneic, Allogeneic or Semi - Allogeneic Thymus or Thoracic Duct Cells

Group and operation at birth	Inoculum	Sheep erythrocyte challenge	No. of mice	Average No. of PFC* per spleen at 4-5 days (±SE)	P values (cf. group 5
1. Tx*	-	-	5	106 ± 24	-
2. STx*	-	-	9	123 ± 29	-
3. Tx	10×10^6 CBA thymus cells	-	6	241 ± 55	-
4. Tx	10×10^6 CBA thoracic duct cells	-	4	323 ± 87	-
5. Tx	-	+	16	2,356 ± 537	-
6. Tx	10×10^6 CBA thymus cells	+	20	19,160 ± 3,840	< 0.01
7. Tx	50×10^6 CBA thymus cells	+	22	38,855 ± 7,448	< 0.01
8. Tx	50×10^6 C57BL thymus cells	+	11	17,448 ± 3,901	< 0.01
9. Tx	10×10^6 (CBA x C57BL)F_1 thymus cells	+	10	22,380 ± 6,285	< 0.01
10. Tx	10×10^6 CBA thoracic duct cells	+	12	20,254 ± 3,646	< 0.01
11. Tx	1.5×10^6 CBA thoracic duct cells	+	5	3,510 ± 1,040	NS
12. Tx	10×10^6 C57BL thoracic duct cells	+	10	30,120 ± 7,791	< 0.01
13. Tx	10×10^6 (CBA x C57BL)F_1 thoracic duct cells	+	12	24,537 ± 5,519	< 0.01
14. STx	-	+	36	32,177 ± 3,550	< 0.01

* Abbreviations: PFC = plaque-forming cells; Tx = thymectomy; STx = sham-thymectomy.

thoracic duct cells and thymus cells are presented in Table 7. It is apparent that the elevation, over and above the number of PFC in thymectomized mice injected with SRBC only, was statistically significant. 10 million thoracic duct cells from these 2 donor types were as effective as syngeneic cells but 50 million C57BL thymus cells did not effect the appearance of as many PFC as 50 million syngeneic thymus cells.

Large-scale experiments to test the immunological activity of spleen cells were not performed. In a single experiment 50×10^6 C57BL spleen cells were injected, together with SRBC, into 2 neonatally-thymectomized CBA mice. The numbers of PFC in the spleens 5 days later were 29, 350 and 22, 400. In another experiment, (CBA x C57Bl) F_1 spleen cells also increased the 5 day PFC response to SRBC. These spleens, together with those from mice injected with C57BL or (CBA x C57BL) F_1 thoracic duct or thymus cells, were used in the PFC identification experiments to be described in the next section.

(c) The identity of the PFC in reconstituted mice. Since C57BL and (CBA x C57BL) F_1 hybrid lymphoid cells increased the SRBC reactivity of neonatally-thymectomized mice, anti-H_2 sera can be used to determine the immunogenetic identity of the antibody-producing cells in the spleens. Using such a technique one should be able to test the hypothesis that the inoculated cells had provided the precursors of the PFC.

The capacity of isoantisera to specifically inhibit the appearance of plaques was first tested with spleen cells from intact CBA, C57BL and (CBA x C57BL) F_1 mice injected 4 days previously with SRBC. CBA and C57BL spleen cells containing PFC were incubated in vitro with normal CBA, normal C57BL, anti-CBA and anti-C57BL sera. (CBA x C57BL) F_1 hybrid spleen cells were incubated, in addition, with normal F_1 serum. Incubation with normal mouse sera caused a reduction in the number of PFC in spleen cell aliquots of from 0 to 49% with an average of 29% (22 determinations). These losses occurred

quite randomly and were presumably a result of non-specific factors operating during the incubation and washing procedures. In each experiment, the number of PFC remaining after incubation with either specific or nonspecific antiserum was expressed as the percent reduction over that obtained with normal mouse sera in the same experiment. The results of a series of experiments, using various batches of antisera (Table 8), indicate that anti-CBA sera caused a reduction of 84 to 91% in the number of CBA PFC in contrast to a 0 to 17% reduction in the number of C57BL PFC. Anti-C57BL reduced the number of C57BL PFC by 83 to 99% and the number of CBA PFC by 0 to 26%. Anti-CBA and anti-C57BL sera both inhibited 91 to 99% of (CBA x C57BL)F_1 PFC. The capacity of isoantisera to discriminate between CBA and C57BL PFC in a mixture of cells was next tested. Spleen cell suspensions containing 330 CBA PFC and 221 C57BL PFC were incubated with anti-C57BL and anti-CBA sera. The number remaining after incubation with anti-CBA serum was 205 and the number remaining after anti-C57BL serum was 285. Hence, each antiserum had reduced the number of PFC by a factor commensurate with the known number of PFC contained in the mixture.

Spleen cells from allogeneically-and semi-allogeneically-reconstituted neonatally-thymectomized CBA mice were incubated with normal mouse sera, anti-CBA, or anti-C57BL sera. The results of 12 experiments are shown in Table 9. Various numbers of mice were sacrificed and the spleens harvested at 4 to 5 days after lymphoid cell inoculation and SRBC challenge. Anti-CBA serum inhibited from 86 to 96% of the PFC in the spleen of mice reconstituted with 10 to 50 million C57BL thymus, thoracic duct or spleen cells but anti-C57BL serum only inhibited from 0 to 12% of the PFC. Similarly, in neonatally-thymectomized mice reconstituted with 10 to 50 million (CBA x C57BL)F_1 hybrid thymus, spleen or thoracic duct cells, anti-CBA (which is directed against both donor and host histocompatibility antigens) caused a reduction of 89 to 97% in the number of PFC whereas

Table 8

Number of Plaque-Forming Cells (PFC) from Spleens of Normal Immunized Mice Remaining after Incubation with Isoantisera

Spleen cell donor	No. of PFC per aliquot remaining after incubation with:-		
	Normal mouse sera	Anti-CBA serum	Anti-C57BL serum
CBA	310	50 (84%)⁂	407 (0%)
	662	68 (90%)	550 (17%)
	529	48 (91%)	394 (26%)
	118	17 (86%)	131 (0%)
	118	125* (0%)	106* (10%)
	ND**	38	628
C57BL	317	360 (0%)	17 (95%)
	442	367 (17%)	74 (83%)
	1,566	1,350 (14%)	162 (90%)
	2,118	ND	28 (99%)
	ND	2,353	87
	ND	2,118	28
(CBA x C57BL)F_1	2,481	16 (99%)	41 (98%)
	815	31 (96%)	44 (95%)
	610	54 (91%)	46 (92%)
	ND	72	42

⁂ Number in brackets refers to percent reduction relative to the value obtained after incubation with normal mouse sera.

* Spleen cell aliquot incubated with antisera in the absence of guinea pig serum.

** No determination or accident.

Table 9

Number of Plaque-Forming Cells (PFC) from Spleens of Reconstituted Neonatally Thymectomized CBA Mice Remaining after Incubation with Isoantisera

Cells used for reconstitution	No. of spleens in pool	No. of PFC per aliquot remaining after incubation with:-		
		Normal mouse sera	Anti-CBA serum	Anti-C57BL serum
C57BL thymus cells	5	460	37 (92%)⁂	407 (12%)
C57BL thymus cells	3	363	36 (90%)	334 (8%)
C57BL spleen cells	2	1,536	126 (92%)	1,512 (2%)
C57BL thoracic duct cells	4	1,612	60 (96%)	1,930 (0%)
C57BL thoracic duct cells	6	142	20 (86%)	140 (1%)
(CBA x C57BL)F_1 thymus cells	3	609	20 (97%)	571 (6%)
(CBA x C57BL)F_1 spleen cells	2	880	127 (85%)	892 (0%)
(CBA x C57BL)F_1 thoracic duct cells	6	130	7 (95%)	108 (17%)
(CBA x C57BL)F_1 thoracic duct cells	4	744	84 (89%)	958 (0%)
(CBA x C57BL)F_1 thoracic duct cells	2*	1,174	29 (98%)	1,454 (0%)
(CBA x C57BL)F_1 thoracic duct cells	3*	90	0 (100%)	80 (11%)
(CBA x C57BL)F_1 thoracic duct cells	3*	817	7 (99%)	796 (3%)

⁂ Number in brackets refers to percent reduction relative to the value obtained after incubation with normal mouse sera.

* Reconstituted mice killed 7 days after cell injection; all others were killed 4 - 5 days after cell injection.

only 0 to 17% were affected by anti-C57BL serum. It can be seen in Figure 18 (C), that the peak response of neonatally-thymectomized CBA mice injected with F_1 thoracic duct cells and SRBC occurred on day 7 after inoculation. Spleens taken at this time contained PFC which were reduced by anti-CBA serum to the extent of 98-100% (Table 9) but which were largely unaffected by anti-C57BL serum (0 - 11% reduction). All the data indicate that isoantisera directed against the histocompatibility antigens of the host were effective in reducing the number of PFC whereas antisera directed specifically against the donor cell type were largely ineffective.

(d) Activity of various cellular and noncellular inocula. Since the majority, if not all, of thePFC in mice reconstituted with allogeneic lymphoid cells were derived from a cell type resident in the neonatally-thymectomized host, it was necessary to test the reconstitutive capacity of other inocula. Accordingly, 3 to 5 weeks old neonatally-thymectomized CBA mice were injected with SRBC and syngeneic and semiallogeneic thymus and thoracic duct cells which had received 1,000 rads x-irradiation in vitro. Irradiated cells failed to significantly elevate the response of the recipient mice to SRBC (Table 10). Cell-free thymus extracts, prepared according to the method of Metcalf (1956), were also ineffective when injected intraperitoneally at the time of SRBC challenge. Fifty million cells prepared from commercial baker's yeast were without effect when injected with SRBC and a similar number of syngeneic bone marrow cells caused a slight but insignificant increase. The large standard error of the mean of the latter group of 11 mice was due to the response of one mouse which produced 25,050 PFC in the spleen. This value exceeded all others in the group by at least 18,600. Huge numbers of DA and Wistar rat thymus cells significantly increased the average PFC response but only by a factor of 3. Human thymus cells were totally ineffective and a recent experiment has demonstrated that guinea pig thymus cells uniformly fail to elevate the PFC response

Table 10

Plaque-Forming Cell Response of Neonatally Thymectomized CBA Mice Injected with Sheep Erythrocytes and Various Cellular and Non-Cellular Inocula

Group	Inoculum	Sheep erythrocyte challenge	No. of mice	Average No. of PFC* per spleen at 4-5 days (± SE)	P values (cf. group 1)
1	-	+	16	2,356 ± 537	-
2	50×10^6 irradiated CBA thymus cells	+	4	1,160 ± 615	NS
3	10×10^6 irradiated CBA thoracic duct cells	+	5	3,776 ± 979	NS
4	10×10^6 irradiated (CBA x C57BL)F_1 thoracic duct cells	+	6	4,344 ± 2,540	NS
5	Thymus extract	+	6	1,802 ± 1,155	NS
6	50×10^6 yeast cells	+	6	2,270 ± 450	NS
7	50×10^6 bone marrow cells	+	11	3,814 ± 2,189	NS
8	100×10^6 rat thymus cells	+	11	7,027 ± 1,400	< 0.01
9	100×10^6 human thymus cells	+	10	1,713 ± 576	NS

* PFC = plaque-forming cells.

of neonatally-thymectomized mice injected with SRBC.

(e) Activity of thoracic duct cells from tolerant mice. Thoracic duct lymphocytes from rats specifically tolerant of sheep erythrocytes have been shown to be less effective than thoracic duct lymphocytes from nontolerant donors in adoptively transferring sheep erythrocyte reactivity to irradiated rats (Section IB). It was felt that the use of thoracic duct cells from tolerant donors might shed light on the nature of the reconstitutive activity of lymphoid cell inocula in neonatally-thymectomized mice. Such inocula should disclose whether the inoculated lymphocytes are simply acting as trephocytes in this system.

The degree and specificity of immunological tolerance induced in CBA mice using the cyclophosphamide method of Dietrich and Dukor (1967) were first determined. In mice previously treated with SRBC and cyclophosphamide, the number of PFC appearing 4 to 5 days after a challenge injection of SRBC was approximately 110,000 less than in cyclophosphamide treated controls ($P < 0.001$) (Table 11). By contrast, mice from these two groups produced comparable numbers of PFC in the spleen in response to a challenge injection of horse erythrocytes (HRBC). The degree of cross-reactivity between SRBC and HRBC has been reported to be slight (Radovich and Talmage, 1967) and this is also evident in the results presented in the table. Mice injected with one erythrocyte type failed to produce many PFC against the other erythrocyte type. When SRBC-tolerant and HRBC-tolerant mice were challenged with both SRBC and HRBC a major response occurred only to erythrocytes against which the mice had not been rendered tolerant. Control mice challenged with both sheep and horse erythrocytes produced significantly less anti-SRBC PFC than when challenged with SRBC alone ($P < 0.02$). The response to HRBC, however, was not significantly altered when such mice were challenged with both SRBC and HRBC.

Table 11

Plaque-Forming Cell Response of CBA Mice Previously Injected with Cyclophosphamide with or without Heterologous Erythrocytes and Challenged with Sheep and/or Horse Erythrocytes

Treatment		Challenge erythrocytes (days 23 to 28)	No. of PFC per spleen 4 to 5 days after challenge (± SE)*	
Day 1	Day 2		Anti-SRBC	Anti-HRBC
SRBC	Cyclo	SRBC*	1,939 ± 187 (35)⁂	165 ± 75 (4)
		HRBC	103 ± 28 (6)	23,333 ± 4,609 (12)
		SRBC + HRBC	1,355 ± 390 (11)	22,910 ± 3,982 (11)
HRBC	Cyclo	SRBC + HRBC	38,883 ± 14,970 (6)	967 ± 401 (6)
-	Cyclo	SRBC	116,936 ± 15,652 (21)	283 ± 60 (6)
		HRBC	423 ± 150 (6)	30,264 ± 7,312 (14)
		SRBC + HRBC	70,540 ± 7,678 (15)	34,742 ± 5,349 (15)

* Abbreviations: PFC = plaque-forming cells;
SRBC = sheep erythrocytes (0.1 ml of 20%);
HRBC = horse erythrocytes (0.1 ml of 20%)

⁂ Number in brackets refers to the number of mice in the PFC assays.

The capacity of thoracic duct cells from SRBC-tolerant mice to transfer SRBC reactivity adoptively was tested by injecting 5 or 10 million cells into 3 to 5 weeks old neonatally-thymectomized mice. Other thymectomized mice of the same age received an equivalent number of cells from cyclophosphamide-treated control mice. The groups of mice were challenged with either SRBC or HRBC, or with both antigens simultaneously. The numbers of PFC in the spleens of the thymectomized recipients were determined 4 to 5 days later and the results are presented in Table 12. Thoracic duct cells from mice tolerant of SRBC were inferior to cells from nontolerant mice in this transfer system. Thus, thymectomized mice inoculated with 10 million thoracic duct cells from tolerant mice produced, in their spleens, an average of 11,690 PFC. Ten million thoracic duct cells from control mice elevated the average number of PFC to 65,067. This difference, and also that obtained following injections of 5 million thoracic duct cells from the two donor types, were statistically significant ($P < 0.01$ and $P < 0.001$ respectively). On the other hand, 10 million thoracic duct cells from both tolerant and control mice increased the average response of thymectomized mice challenged with HRBC to values exceeding 30,000 PFC per spleen. By inoculating recipient mice with 10 million thoracic duct cells from SRBC-tolerant mice and challenging with both SRBC and HRBC, the average anti-SRBC PFC response was elevated to 21,106. This value, despite the relatively large group size, was not significantly different from the anti-SRBC PFC response of 11,690 in similar mice challenged with SRBC only ($P < 0.1$). Ten million thoracic duct cells from nontolerant mice, when injected into thymectomized mice together with both SRBC and HRBC, effected the appearance of 57,789 anti-SRBC PFC, this figure being significantly different from the 21,106 PFC per spleen in the group receiving cells from SRBC-tolerant mice ($P < 0.05$). In groups inoculated with thoracic duct cells from tolerant or control mice, the responses to HRBC were not affected by injecting both SRBC and HRBC.

Table 12

Plaque-Forming Cell Response of Neonatally Thymectomized CBA Mice Injected with Heterologous Erythrocytes and Thoracic Duct Cells from either Sheep Erythrocyte-Tolerant or Non-Tolerant Syngeneic Mice

Thoracic duct cell donor	No. of thoracic duct cells injected ($\times 10^{-6}$)	Challenge erythrocytes	No. of recipient mice	No. of PFC per spleen at 4 to 5 days ($\pm$ SE)⁂	
				Anti-SRBC	Anti-HRBC
Non-tolerant	5	SRBC⁂	5	29,590 $\pm$ 2,179	-
	10	SRBC	9	65,067 $\pm$ 14,704	-
	10	HRBC	5	-	36,020 $\pm$ 7,246
	10	SRBC + HRBC	9	57,789 $\pm$ 13,866	33,877 $\pm$ 9,660
Tolerant	5	SRBC	5	6,660 $\pm$ 1,851	-
	10	SRBC	10	11,690 $\pm$ 2,001	-
	10	HRBC	4	-	32,715 $\pm$ 10,682
	10	SRBC + HRBC	17	21,106 $\pm$ 4,457	30,241 $\pm$ 6,275
-	-	SRBC	10	2,555 $\pm$ 659	-
		SRBC*	7	1,657 $\pm$ 1,092	-
		HRBC	6	-	2,812 $\pm$ 1,480
		SRBC + HRBC	7	3,814 $\pm$ 1,660	1,071 $\pm$ 342

⁂ Abbreviations: PFC = plaque forming cells;
SRBC = sheep erythrocytes (0.1 ml of 20%);
HRBC = horse erythrocytes (0.1 ml of 20%)

* 0.5 ml of 50% erythrocyte suspension $\equiv$ 2×10^9 erythrocytes.

By comparing the data in Tables 7 and 12, it is evident that an inoculum of 10 million thoracic duct cells from 12 weeks old cyclophosphamide treated mice was more effective than 10 million cells from normal 6 to 8 weeks old mice in elevating the SRBC responsiveness of neonatally-thymectomized mice. This may be due to the age difference between the 2 donor types, or it may be a consequence of recovery from cyclophosphamide. The PFC response to SRBC in cyclo-treated mice is certainly higher than could be expected in 6 to 8 weeks old mice (Table 11 and reference Weiss et al., 1967).

Thoracic duct cells from SRBC-tolerant mice were inferior to cells from nontolerant mice in transferring reactivity to the specific tolerising antigen. Nevertheless, such inocula significantly increased the PFC response of neonatally-thymectomized mice to SRBC. This was especially so when thymectomized recipients were challenged with both SRBC and HRBC. The difference between the mean of the group not inoculated with lymphoid cells (3,814 PFC) and the mean of the group receiving 10 million thoracic duct cells from SRBC-tolerant mice (21,106 PFC) was highly significant ($P < 0.02$). An additional group was included at this stage. Neonatally-thymectomized mice were injected with approximately 10 times the number of erythrocytes in the standard immunizing dose of SRBC used throughout these experiments. As seen in Table 12, the 4 to 5 day PFC response was not increased above that following the routine injection of 0.1 ml of 20% SRBC ($\equiv 10^8$ SRBC).

(f) Attempts at breaking tolerance with thoracic duct lymphocytes

From the results presented in Tables 11 and 12, it is clear that the difference in the activity of thoracic duct cells from tolerant and control mice in neonatally-thymectomized recipients is not as great as would have been expected from an examination of the difference in the PFC response of the cell donors. Thus the difference in the PFC response to SRBC approached 2 $\log_{10}$ in the donor mice yet only a 6 fold difference

in the PFC response was recorded in neonatally-thymectomized mice injected with 10 million cells from the two donor types. One interpretation of this observation is that tolerance in the donors does not only involve the thoracic duct lymphocyte population. Experiments were then set up to test whether thoracic duct cell inocula from normal mice were effective in breaking SRBC tolerance in SRBC-cyclo treated mice. In addition, the efficacy of bone marrow cells, spleen cells, and a mixture of bone marrow and thoracic duct cells, was determined in other tolerant mice. The inocula were injected intravenously together with sheep erythrocytes and the splenic PFC response measured 5 days later. The results are presented in Table 13.

Twenty million normal thoracic duct cells significantly increased the PFC response in tolerant mice but the increase was slight. Bone marrow cells were ineffective but a mixture of these two cell types increased the PFC number, albeit marginally once again. The finding that huge numbers of spleen cells were also unable to elevate the response to normal levels hints at the possibility that few of the competent cells in the inocula were responding to SRBC in the environment of the tolerant hosts. Many recipient mice in all groups did not produce PFC in numbers above the average response of tolerant mice injected with SRBC alone. By comparing the data in Tables 7 and 13, it is evident that neonatally-thymectomized mice and SRBC-cyclo treated mice were able to respond to SRBC by producing an average of 2,356 PFC and 1,939 PFC at day 5, respectively. Ten million syngeneic thoracic duct cells increased this response in neonatally-thymectomized mice to an average of 20,254 PFC but 20 million syngeneic thoracic duct cells increased the response in tolerant mice to only 3,067 PFC.

(ii) Immunological activity of thymus, thoracic duct, and bone marrow cells in irradiated mice. The experimental system employing reconstituted neonatally-thymectomized mice failed to detect the presence of PFC precursors in thymus, thoracic duct or spleen cell inocula. As

Table 13

Plaque-Forming Cell (PFC) Response of Sheep Erythrocyte-Tolerant CBA Mice Injected with Sheep Erythrocytes (SRBC) and Various Inocula from Normal Syngeneic Mice

Group	Inoculum	No. of mice	No. of PFC 5 days after injection (±SE)	P values (c.f. Group1)
1	SRBC	35	1,939 ± 187	-
2	20×10^6 thoracic duct cells + SRBC	16	3,067 ± 619	< 0.05
3	20×10^6 bone marrow cells + SRBC	9	2,280 ± 650	N.S.
4	20×10^6 thoracic duct cells + 20×10^6 bone marrow cells + SRBC	9	7,617 ± 1,951	< 0.001
5	40×10^6 spleen cells + SRBC	10	7,510 ± 2,739	< 0.001

mentioned previously, discrete foci of haemolysis appeared in the spleens of heavily-irradiated mice injected with SRBC and thoracic duct or spleen cells. ThePFC in this situation must therefore be derived from the inoculated cells or from a radioresistant cell type in host tissues. The following experiments were designed to determine whether PFC precursors are present in cells from the thymus or thoracic duct.

(a) Activity of syngeneic cells. Eight weeks old CBA mice were injected with 10 million thymus cells, bone marrow cells or thoracic duct cells together with SRBC within 6 hours of receiving 800 rads x-irradiation. The CBA donor mice were aged 6 to 8 weeks. Seven to 8 days later the spleens were removed and the number of PFC determined. Thoracic duct cells significantly increased the average PFC response above that obtained in irradiated mice injected with SRBC only ($P < 0.01$) whereas thymus and bone marrow cell inocula were ineffective (Table 14). Moreover, 1 million thymus cells did not increase the number of PFC but the 97 PFC following an injection of 1 million thoracic duct cells was significantly higher than the average number in the control group injected with SRBC only ($P < 0.05$). Increasing the size of the thymus inoculum to 50 million cells did not significantly increase the PFC response and 25 million thoracic duct cells, injected in the absence of SRBC, were also without effect. Varying the dose of SRBC did not influence the negligible PFC-producing capacity of bone marrow cells at 7 to 8 days after injection into irradiated recipients.

It was of interest to examine the differences in the immunological activity of thymus, thoracic duct, and bone marrow cells at a cytological level. Eight weeks old CBA mice were injected with either 50 million CBA/T6T6 thymus cells, 10 million T6T6 thoracic duct cells, or 10 million T6T6 bone marrow cells together with SRBC. Some mice were injected with the three nucleated cell inocula alone. Cytological

Table 14

Number of Plaque-Forming Cells (PFC) in Spleens of Heavily Irradiated CBA Mice after Injection of Sheep Erythrocytes (SRBC) and Syngeneic Thymus Cells, Thoracic Duct Cells and Bone Marrow Cells

Cells inoculated	No. of mice	Average No. of PFC per spleen at 7 - 8 days postirradiation (± SE)
SRBC only	36	18 ± 3.3
1×10^6 thymus cells + SRBC	18	24 ± 9.5
10×10^6 thymus cells + SRBC	14	15 ± 3.0
50×10^6 thymus cells + SRBC	20	52 ± 19.3
1×10^6 thoracic duct cells + SRBC	10	97 ± 34.7
10×10^6 thoracic duct cells + SRBC	23	1,270 ± 338
25×10^6 thoracic duct cells only	5	17 ± 4.4
10×10^6 bone marrow cells + SRBC	34	47 ± 11.1
10×10^6 bone marrow cells + SRBC ✱	6	20 ± 8.9
10×10^6 bone marrow cells + SRBC ✱✱	9	17 ± 7.5
10×10^6 bone marrow cells + SRBC ✱✱✱	8	14 ± 11.1

✱ 1×10^6 SRBC; ✱✱ 1×10^7 SRBC; ✱✱✱ 1×10^9 SRBC.
Other doses of SRBC = 1×10^8

analyses were performed at 3, 4, 5, 6 and 7 days after irradiation and cell injection and the numbers of mitotic figures per 2,000 nucleated cells in the spleen cell smears are shown in Figure 19. Of 324 well-spread mitotic figures in spleen cell smears from thymus-and thoracic duct-incubating hosts only 7 could not be unequivocally identified as being of CBA/T6T6 type. No mitoses were found at any time in 10 irradiated mice injected with SRBC only. It can be seen in Figure 19 that no mitoses were detected in hosts of 50 million thymus cells but a few were present in the spleens of hosts incubating thoracic duct cells. The addition of SRBC increased the number of mitoses/2,000 cells to peak values of 8 and 20 for thymus- and thoracic duct cell-incubating hosts, respectively. Interestingly, mitotic figures were still present at day 7 when PFC might be expected (Table 14) but they were present in high numbers only in the mice injected with thoracic duct cells and SRBC. Mitoses were numerous on day 4 to 7 following bone marrow injection and little difference was apparent whether or not SRBC were included in the inoculum. Evidence of haemopoietic regeneration was apparent on day 5 when the spleens were visibly increased in size and by day 7 the spleens appeared to be of normal size. By contrast, no signs of haemopoietic regeneration, including macroscopic colonies (Till and McCulloch, 1961), were detected in the spleens of either thymus- or thoracic duct cell-injected mice. The difference in the total number of splenic mitotic figures between bone marrow-incubating hosts and all others must thus be far greater than is indicated by the data on the number of mitoses/2,000 cells.

(b) Activity of semiallogeneic thoracic duct cells and identity of PFC. SRBC and 6 to 30 million thoracic duct cells from (CBA x C57BL)F_1 hybrid mice were injected into CBA mice which had received 800 rads total body x-irradiation. The PFC in the spleens 7 days later were enumerated and aliquots of the spleen cell suspensions

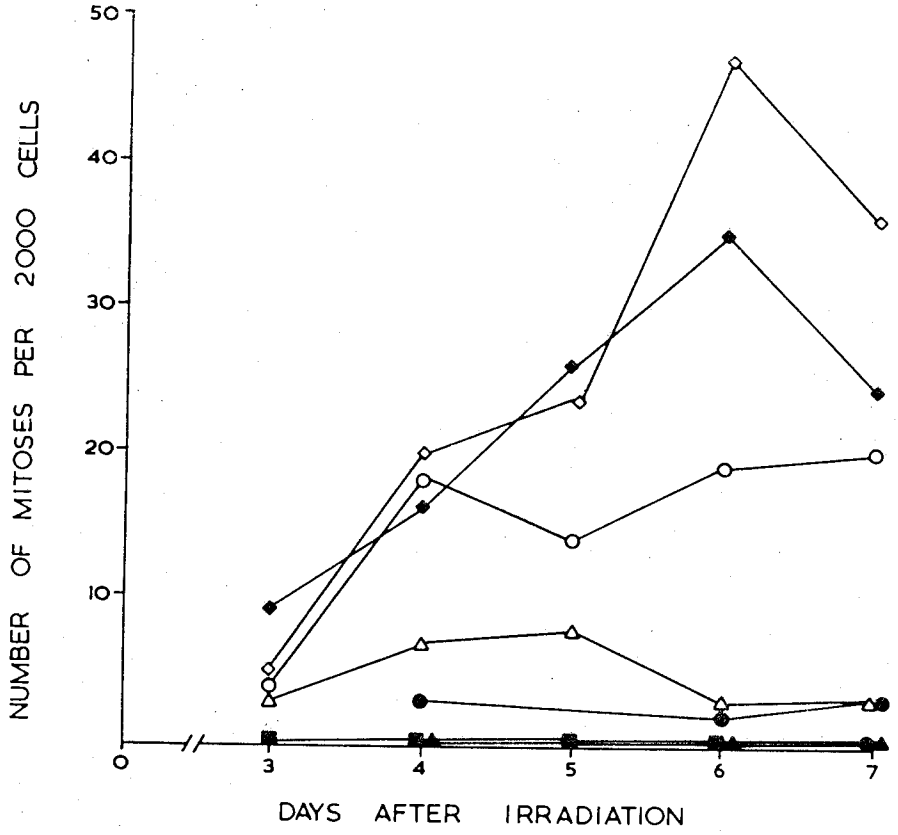


Figure 19. Number of dividing cells at 3 to 7 days postirradiation in the spleens of heavily irradiated CBA mice injected with SRBC only (■), 50 million CBA/T6T6 thymus cells only (▲), 10 million CBA/T6T6 thoracic duct cells only (●), 10 million CBA/T6T6 bone marrow cells only (◆), thymus cells + SRBC (△), thoracic duct cells + SRBC (O), and bone marrow cells + SRBC (◇). Each point represents the number of mitoses counted in smears prepared from a single spleen.

incubated with normal mouse sera and anti-CBA and anti-C57BL isoantisera. It is evident in Table 15 that both isoantisera reduced the number of PFC appearing in the spleen by 85 to 95%. The PFC in this situation are therefore derived from the inoculated thoracic duct cells.

(c) Interaction between thoracic duct cells and bone marrow cells in irradiated mice. During the haemolytic focus assays for the detection of ARC in lymphoid populations, it was noticed that the foci resulting from spleen cell inocula extended over more slices than was the case following injections of thoracic duct cells. A most obvious difference between the spleens of irradiated mice injected with either of these two cell types was that myeloid regeneration had occurred after spleen cell injection but not after thoracic duct cell injection. The effect of myeloid regeneration on the size of "thoracic duct foci" was determined in preliminary experiments using (CBA x C57BL)F_1 mice. One million thoracic duct cells and/or 10 million bone marrow cells were injected on the day of irradiation into syngeneic recipients which had received 800 rads x-irradiation. The numbers of foci appearing in response to SRBC in the spleens of recipients of the mixture were not increased over those in recipients of thoracic duct cells (Table 16). Other mice had their spleens assayed for PFC. The average number of PFC in the spleens of mice receiving thoracic duct and bone marrow cells was far greater than that in spleens of mice injected with either thoracic duct or bone marrow cells alone.

The phenomenon was then studied more thoroughly in CBA mice. Groups of irradiated 8 weeks old CBA mice were injected with SRBC together with either 1 million syngeneic thoracic duct cells, 10 million bone marrow cells, or a mixture of both cell types. Mice were killed 4, 5, 6, 7, 8 and 9 days after irradiation and the spleens assayed for their content of PFC or haemolytic foci. The results of these time

Table 15

Plaque-Forming Cells (PFC) Remaining after Incubation of Spleen Cells from Heavily Irradiated CBA Mice Injected with Sheep Erythrocytes (SRBC) and (CBA x C57BL)F_1 Thoracic Duct Cells

Inoculum	No. of spleens in pool	Average No. of PFC in spleen at 7 days	No. of PFC remaining after incubation with:-		
			Normal mouse sera	Anti-CBA serum	Anti-C57BL serum
SRBC only	10	19	-	-	-
6×10^6 thoracic duct cells + SRBC	3	593	98	14 (85%)	11 (89%)
10×10^6 thoracic duct cells + SRBC	7	822	72	6 (92%)	11 (85%)
30×10^6 thoracic duct cells + SRBC	4	5,655	432	32 (93%)	21 (95%)

Table 16

Haemolytic Foci and Plaque-Forming Cells (PFC) Produced in the Spleens of Heavily Irradiated (CBA x C57BL)F_1 Mice after Injection of Sheep Erythrocytes (SRBC) and Syngeneic Thoracic Duct Cells and/or Bone Marrow Cell

Inoculum	Haemolytic foci 8 days postirradiation		PFC 8 days postirradiation	
	No. of mice	No. of foci per spleen (Individual No. $\pm$ or Average No. $\pm$ SE)	No. of mice	Average No. of PFC per spleen ($\pm$SE)
SRBC only	10	0, 0, 0, 0, 0, 0, 0, 1, 1, 1	8	12 ± 3.5
10×10^6 bone marrow cells + SRBC	4	0, 0, 0, 1	4	31 ± 2.9
1×10^6 thoracic duct cells + SRBC	12	5.3 ± 0.5	13	44 ± 12.3
1×10^6 thoracic duct cells + 10×10^6 bone marrow cells + SRBC	13	5.2 ± 0.7	16	527 ± 75.4

course studies are shown in Figures 20 and 21. Since thoracic duct inocula do not contain haemopoietic stem cells, recipients of such cells were destined to die of haemopoietic failure. The data for day 9 in the group of mice injected with thoracic duct cells and SRBC were therefore incomplete and are not shown in the figures. The average peak number of haemolytic foci following thoracic duct cell injection was 5 and this was not increased significantly by a simultaneous injection of bone marrow cells. At the peak of the response (day 8), the average number of foci produced by thoracic duct cells or the mixed inoculum was significantly greater than that produced by bone marrow cells ($P < 0.001$). Ten million bone marrow cells effected the appearance of < 2 haemolytic foci on all days after irradiation and the mean number at the peak was not significantly above the background number of haemolytic foci in mice injected with SRBC only (Figure 20). Bone marrow therefore lacks focus-producing cells and it does not increase the number of foci resulting from an injection of thoracic duct cells.

It can be seen in Figure 21 that either 1 million thoracic duct cells or 10 million bone marrow cells produced about 100 PFC in the spleens of irradiated mice in response to SRBC. By contrast, recipients of a mixture of these 2 cell types, contained approximately 900 PFC in the spleen at the peak of the response. This figure was significantly different from the maximum average number of PFC in the spleens of recipients of either bone marrow cells or thoracic duct cells ($P<0.01$). If each haemolytic focus in the spleen represents a cluster of PFC then it can be calculated from Figures 20 and 21 that bone marrow cells had increased the number of PFC per focus in the spleens of thoracic duct cell-inoculated mice from < 20 to 160.

(d) Interaction between combinations of semiallogeneic cells in irradiated mice. Semiallogeneic cells were next tested for their capacity to act synergistically in irradiated mice in the production of PFC, the aim being to use anti-H_2 sera in the identification of the

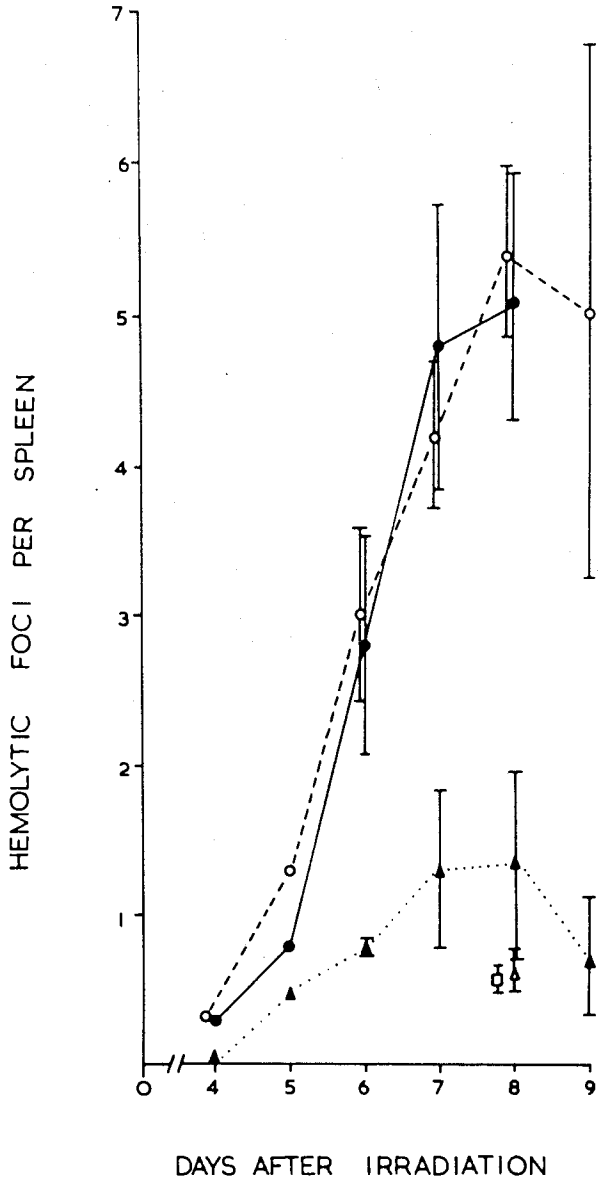


Figure 20. Haemolytic foci produced in the spleens of heavily irradiated CBA mice injected on the day of irradiation with sheep erythrocytes (SRBC) only (Δ), syngeneic thoracic duct cells only (□), or a combination of SRBC and either 1 million syngeneic thoracic duct cells (●——●), 10 million syngeneic bone marrow cells (▲·····▲), or a mixed inoculum of 1 million syngeneic thoracic duct cells and 10 million syngeneic bone marrow cells (O-----O). The magnitude of twice the standard error of each mean is shown by the vertical bars. Each point at 4, 5 and 9 days represents the mean of determinations made on 2-4 mice and at 6, 7 and 8 days on 6 - 14 mice.

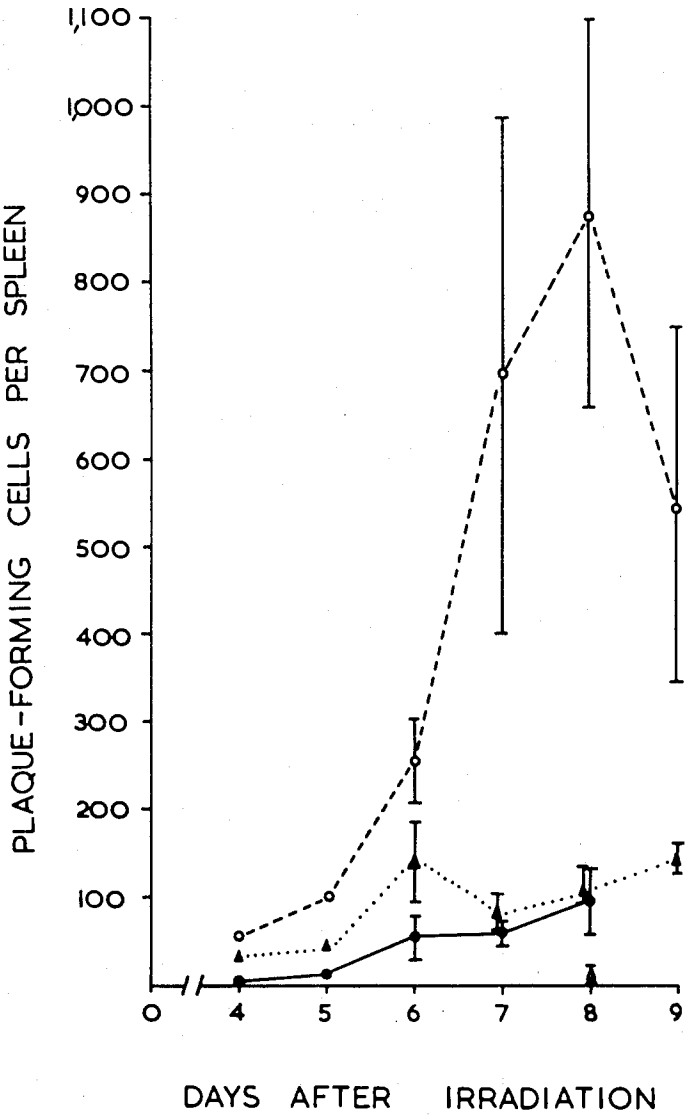


Figure 21. Plaque-forming cells produced in the spleens of heavily irradiated CBA mice injected on the day of irradiation with sheep erythrocytes (SRBC) only (Δ), or a combination of SRBC and either 1 million syngeneic thoracic duct cells (●——●), 10 million syngeneic bone marrow cells (▲·····▲), or a mixed inoculum of 1 million syngeneic thoracic duct cells and 10 million syngeneic bone marrow cells (O-----O). The magnitude of twice the standard error of each mean is shown by the vertical bars. Each point at 4, 5 and 9 days represents the mean of determinations made on 2 - 5 mice and at 6, 7 and 8 days on 6-13 mice.

resulting PFC. It was felt that these experiments should provide an answer to the question of whether bone marrow cells promote the differentiation of PFC from precursors in the thoracic duct cell population or whether they themselves provide such precursor cells. Accordingly, 8 to 10 week old CBA or (CBA x C57BL)F_1 mice received 800 rads x-irradiation and were injected with 10 million CBA bone marrow cells, 1 to 6 million F_1 thoracic duct cells, and SRBC. This particular combination of cells was chosen to reduce the risk of an immunological reaction between the cells of the mixture and also against the irradiated host. Bone marrow cells rather than thoracic duct cells were always of CBA type, since they are much less active in graft-versus-host reactions (Section IC). In a series of experiments no synergism in the production of PFC was detected in irradiated mice injected 7 to 8 days previously with mixtures of semi-allogeneic cells. Allowing the bone marrow to reside in the irradiated host for a period of 2 days prior to injecting the F_1 thoracic duct cells and SRBC did not result in any interaction and again the activity of the thoracic duct-bone marrow cell combination could be accounted for by summating the activities of thoracic duct cells and bone marrow cells alone.

The adult-thymectomized, irradiated and bone marrow protected mouse was then chosen as a host in order to test the activity of combinations of semiallogeneic cells in the irradiated mouse environment. CBA mice were thymectomized at 6 weeks of age and 1 to 3 weeks later were irradiated (800 rads) and injected with 2 to 5 million CBA bone marrow cells. Two weeks after irradiation the mice were injected with 10 million thoracic duct or thymus cells from (CBA x C57BL)F_1 mice together with SRBC. Other recipients received SRBC and syngeneic thymus or thoracic duct cells. The numbers of PFC appearing in the spleens were determined 2, 4, 5, 7, and 10 days later. The results are plotted in Figures 22 and 23. The average response of adult-thymectomized, irradiated CBA mice incubating CBA bone marrow

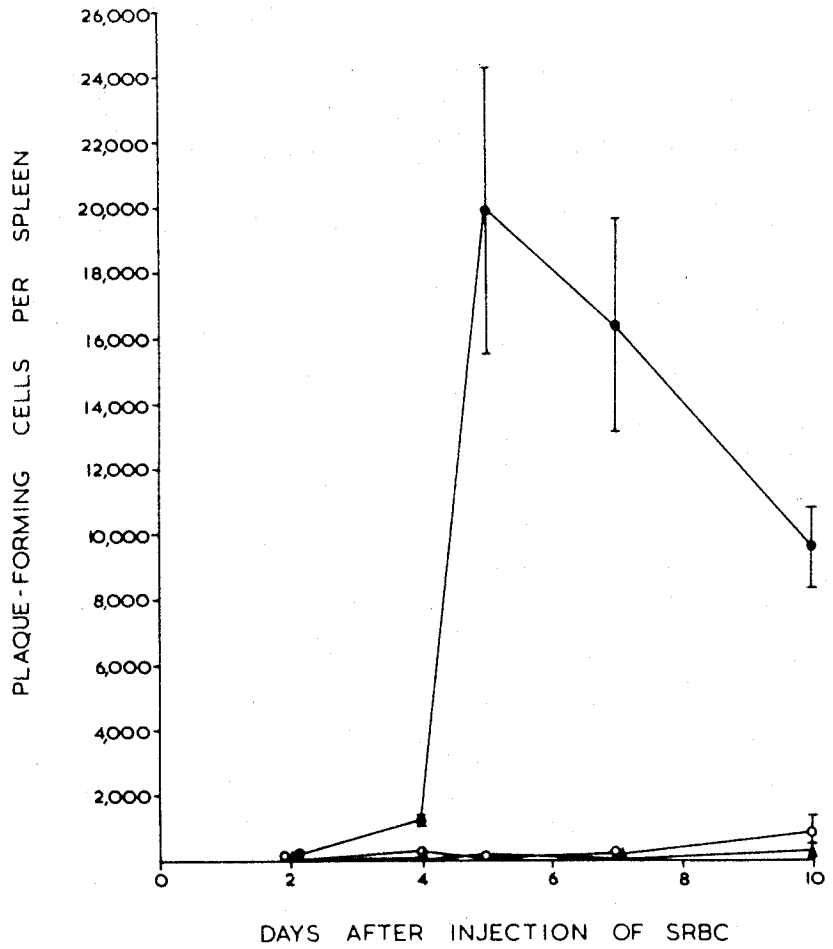


Figure 22. Number of plaque-forming cells in the spleens of heavily irradiated adult thymectomized CBA mice injected on the day of irradiation with syngeneic bone marrow cells followed two weeks later by injections of SRBC only (▲——▲), 10 million (CBA x C57BL)F_1 thymus cells and SRBC (O——O), or 10 million (CBA x C57BL)F_1 thoracic duct cells and SRBC (●——●). The magnitude of twice the standard error of each mean is shown by the vertical bars and each point represents the mean of determinations made on 2 to 10 (average 5) mice.

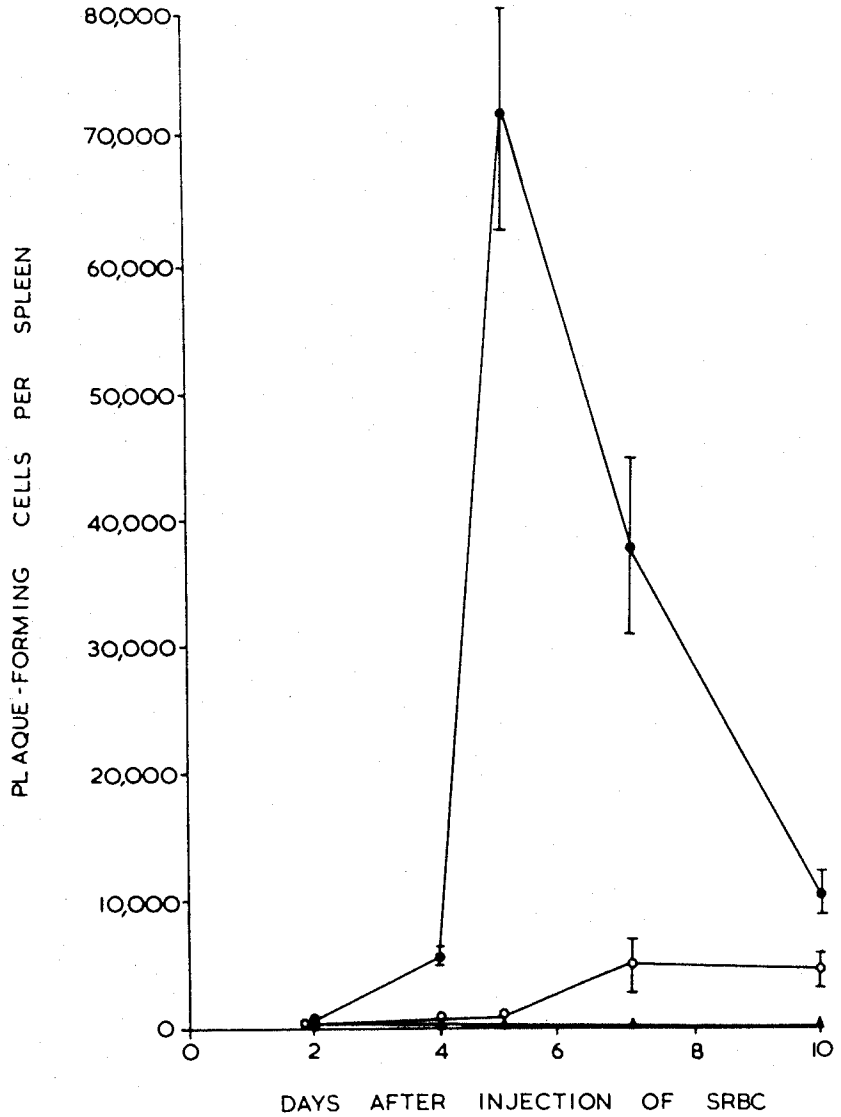


Figure 23. Number of plaque-forming cells in the spleens of heavily irradiated adult thymectomized CBA mice injected on the day of irradiation with syngeneic bone marrow cells followed two weeks later by injections of SRBC only (▲——▲), 10 million syngeneic thymus cells and SRBC (○——○), or 10 million syngeneic thoracic duct cells and SRBC (●——●). The magnitude of twice the standard error of each mean is shown by the vertical bars and each point represents the mean of determinations made on 3 to 10 (average 5.3) mice.

for two weeks was < 300 PFC per spleen at all time points following SRBC injection. Semiallogeneic thymus cells were totally ineffective in elevating the PFC response to SRBC. Syngeneic thymus cells resulted in an average response of 5,756 PFC per spleen on day 7, this value being not significantly different from the average peak number after SRBC injection ($P < 0.1$). Ten million CBA or F_1 thoracic duct cells increased the number of PFC per spleen to average peak values, on day 5, of 72,037 and 19,800 respectively. Hence, the number of PFC following an injection of 10 million thoracic duct cells and SRBC was far greater in adult-thymectomized, irradiated mice injected with CBA bone marrow than in irradiated mice not receiving bone marrow (Figures 22 and 23, c.f. Tables 14 and 15). The superiority of thoracic duct cells over thymus cells in the adult-thymectomized, irradiated and marrow protected hosts contrasts with the situation in neonatally-thymectomized mice (Table 7). One difference between these two host types is in the size of the spleen. Thus, in 17 neonatally-thymectomized 3 to 4 weeks old mice the spleen contained 148 ± 12.2 million nucleated cells whereas in 7 thymectomized radiation chimaeras, the spleen contained 81 ± 7.0 million cells two weeks after irradiation. It is conceivable, therefore, that more injected thymus cells were trapped and recruited in the spleen of the neonatally-thymectomized mouse than in the spleen of the irradiated chimaeric host. This possibility will be discussed in Section III.

Thymus and thoracic duct cells were tested for their capacity to elevate the PFC response in thymectomized radiation chimaeras at 4 and 6 weeks after irradiation and marrow protection. As can be seen in Table 17, thymus cells were vastly inferior to thoracic duct cells at 4 and 6 weeks after establishment of the chimaera. Thymus cells increased the response of well-established chimaeras above that which could be expected in SRBC-injected hosts (Figure 13) but this increase was significant only at 4 weeks ($P < 0.01$).

Table 17

Plaque-Forming Cell (PFC) Response of Adult Thymectomized CBA Mice Injected with Sheep Erythrocytes (SRBC) and Syngeneic Thymus or Thoracic Duct Cells at Various Times after Irradiation and Bone Marrow Protection

Weeks after irradiation and marrow protection	Inoculum	No. of mice	Average No. of PFC per spleen at 5 days (± SE)	P values
2	10×10^6 thymus cells + SRBC	7	814 ± 160	< 0.001
	10×10^6 thoracic duct cells + SRBC	9	$72,037 \pm 8,877$	
4	10×10^6 thymus cells + SRBC	5	$6,820 \pm 961$	< 0.05
	10×10^6 thoracic duct cells + SRBC	4	$73,750 \pm 21,820$	
6	10×10^6 thymus cells + SRBC	8	$10,800 \pm 2,112$	< 0.01
	10×10^6 thoracic duct cells + SRBC	7	$84,386 \pm 17,910$	

(c) Identity of PFC in thymectomized radiation chimaeras. Aliquots of spleen cells were taken from adult-thymectomized, irradiated mice injected with CBA bone marrow, (CBA x C57BL)F_1 thoracic duct cells, and SRBC, and incubated with isoantisera in the manner previously described. Ten or 35 million F_1 thoracic duct cells had been injected together with SRBC two weeks after irradiation and marrow protection. The results of incubating the spleen cell aliquots with the various sera are shown in Table 18. Anti-CBA sera (directed against both thoracic duct and bone marrow cell donor types) caused a reduction in the number of PFC, relative to that following incubation with normal sera, of from 95 to 97%. Incubation with anti-C57BL sera resulted in losses of up to 5%. The majority of PFC produced in these hosts were not of F_1 type and must therefore be CBA in type.

The antisera data indicate that either the CBA bone marrow or the irradiated CBA host had provided the precursors of PFC in syngeneic CBA radiation chimaeras injected with F_1 thoracic duct cells and SRBC. The restoration of immunological reactivity on the part of the host is rendered unlikely by the finding that these hosts, when not injected with thoracic duct cells, were unable to respond to SRBC by producing PFC in the spleen. To test whether the host was contributing PFC precursors, 7 weeks old (CBA x C57BL)F_1 mice were thymectomized and irradiated 2 weeks later. Immediately after irradiation 5 to 6 million CBA bone marrow cells were injected followed 2 weeks later by 10 million (CBA x C57BL)F_1 thoracic duct cells and SRBC. PFC in the spleen were enumerated 5 days later and aliquots of the spleen cells incubated with normal CBA, C57BL and F_1 sera and the two isoantisera. The results of 3 experiments are included in Table 18. In only one of three experiments was the number of PFC increased to levels attained in syngeneic radiation chimaeras. Nevertheless, PFC numbers in two of

Table 18

Plaque-Forming Cells (PFC) Remaining after Incubation of Spleen Cells from Adult Thymectomized, Irradiated CBA or (CBA x C57BL)F_1 Mice Injected with CBA Bone Marrow Cells, (CBA x C57BL)F_1 Thoracic Duct Cells, and Sheep Erythrocytes (SRBC)

Host strain	Inoculum	No. of spleens in pool	Average No. of PFC per spleen	No. of PFC remaining after incubation with:-		
				Normal mouse sera	Anti-CBA serum	Anti-C57BL serum
CBA	SRBC	5	158	-	-	-
	10×10^6 F_1 thoracic duct cells + SRBC	5	10,980	80	4 (95%)*	88 (0%)
	10×10^6 F_1 thoracic duct cells + SRBC	5	18,262⁂	139	6 (96%)	140 (0%)
	35×10^6 F_1 thoracic duct cells + SRBC	2	69,300	604	20 (97%)	571 (5%)
	35×10^6 F_1 thoracic duct cells + SRBC	2	85,850	1,454	70 (95%)	1660 (0%)
(CBA x C57BL)F_1 (1)	SRBC	2	100	-	-	-
	10×10^6 F_1 thoracic duct cells + SRBC	4	925	-	-	-
(2)	SRBC	1	260	-	-	-
	10×10^6 F_1 thoracic duct cells + SRBC	3	18,633	624	18 (96%)	473 (24%)
(3)	SRBC	3	130	-	-	-
	10×10^6 F_1 thoracic duct cells + SRBC	6	2,288	65	0 (100%)	57 (12%)

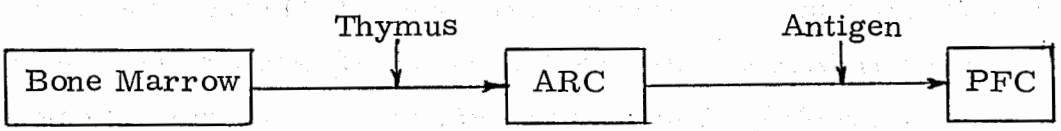
* Number in brackets refers to the percent reduction relative to the value obtained after incubation with normal mouse sera.

⁂ Spleens assayed 7 days after thoracic duct cell injection; all others were assayed 5 days after cell injection.

the three experiments were increased substantially above the number following injection of SRBC only. Since only 12 and 24% of the PFC were inhibited by anti-C57BL serum, the majority must have been of CBA type and thus derived from the bone marrow inoculum.

III. DISCUSSION

It is evident from the experimental results presented in this dissertation that the term "sheep erythrocyte antigen-reactive cell" (ARC) can no longer be used in its original form and must therefore be clearly defined. Kennedy et al. (1965 a, 1966) proposed that the ARC was a founder cell in a clone of haemolysin plaque-forming cells (PFC). In irradiated mice this cell was assumed to localise in the spleen, react to antigen or processed antigen, and produce a progeny of PFC through mechanisms involving several sequential mitotic divisions associated with differentiation. This concept led to the construction of the following minimal model of thymus-influenced immunogenesis, expressed in terms of a differentiative cell lineage:



As mentioned in Section IIA, this scheme was the basic working model behind many of the early experiments described in this thesis.

Two assumptions implicit in the Kennedy definition have yet to be proven. Firstly, it has not been shown that the haemolytic foci in the Kennedy assay represent the sites of PFC clones. The data of Cunningham (1968b) and Kind and Campbell (1968) indicate that the distribution of PFC in the spleens of irradiated mice is not nearly as sharply defined as the focus assay suggests. Secondly, the relationship between the cells responding to sheep erythrocytes (SRBC) and the resultant population of PFC has not been determined. The cell which initiates focus formation may or may not be of the same cell lineage as the PFC. If the precursors of the PFC do not respond to antigen and if the cells which respond to SRBC do not produce progeny PFC then

neither cell type qualifies for the name antigen-reactive cell. It is because of these considerations that the term "focus-forming-cell" (FFC, Gregory and Lajtha, 1968) will be substituted for ARC in those discussions in which the focus-producing capacity of lymphoid cell populations is under examination.

The most obvious histological consequence of thymectomy in the mouse is a reduction in the number of small lymphocytes in the blood and in organs such as the spleen and lymph nodes. These sites are known to be within the "orbit" traversed by recirculating small lymphocytes (Section IA). The thoracic duct cannulation experiments have demonstrated that the thoracic duct lymphocyte population in young adult mice is indeed affected by the absence of the thymus from birth. The lymphocyte deficiency recorded here is of the same order of magnitude as that reported in neonatally-thymectomized rats. Agnew (1967), Rieke and Schwarz (1967) and Goldschneider and McGregor (1968a) noted a reduction of up to 75% in the number of thoracic duct lymphocytes drained over periods of 12 to 72 hours from young adult neonatally-thymectomized Lewis rats. Moreover, Rieke and Schwarz (1967) calculated from the results of their tritiated thymidine studies (Section IA) that the deficiency in the thoracic duct lymphocyte population following neonatal thymectomy in the rat was restricted to the long-lived cells. These quantitative studies on the thoracic duct lymphocyte population have substantiated the claims of the histologists that one of the hallmarks of neonatal thymectomy is a reduction in the number of long-lived, recirculating small lymphocytes.

The chronic output determinations have demonstrated quite clearly that thymectomy in adult life is not followed by a dramatic fall in the number of thoracic duct small lymphocytes until many months after

thymectomy. This finding contrasts with the data of Schooley and Shrewsbury (1967) in which a 50% reduction in lymphocyte number was apparent 3 days after adult thymectomy. Their experiments differ from those reported here in that measurements were made over drainage periods of only 10 to 15 minutes in anaesthetized mice.

Lymph-borne lymphocytes increase in number soon after birth (Heath 1964, Morris 1968) and small lymphocytes are not plentiful in the spleen and lymph nodes of neonatal mice (Archer et al. 1964). The difference in the number of lymphocytes in the thoracic duct of neonatally-thymectomized and adult-thymectomized mice suggests that the effects of thymectomy are more pronounced when performed before the appearance of significant numbers of recirculating lymphocytes. Once the pool has been established, no immediate effect on cell numbers can be expected to result from thymectomy. It must be emphasized, however, that, although the 5 to 6 weeks old neonatally-thymectomized mice were of normal body weight, they were presumably destined to die of wasting disease within 6 weeks or so. Intercurrent infections, by means of some unknown mechanism, may therefore contribute to the paucity of lymphocytes in the recirculating pool of such mice.

Changes in the 48 hour "mobilizable pool" are reflected in the splenic PFC response to SRBC and the correlation between the two measurements is quite striking. The peak PFC response in young neonatally-thymectomized CBA mice is reduced when compared with that in sham-operated controls by 93%, the output of lymphocytes by 98%. In two years old CBA mice the PFC response is reduced over that in two months old mice by about 50% (Wigzell and Stjernwärd, 1966; Aisenberg and Davis, 1968), the output of lymphocytes by about 60%. The SRBC responsiveness of young adult-thymectomized mice is normal (Metcalf, 1965; Miller, 1965; Aisenberg and Davis, 1968) but the PFC number is

reduced to about 25% of that in sham adult-thymectomized CBA mice at 2 years of age (Aisenberg and Davis 1968). At this time the 48 hour pool is reduced in size to about 40% of that in sham-operated controls.

After an irradiation-induced depression in the thoracic duct lymphocyte number, the pool is increased perceptibly in size only at, or later than, 4 weeks after irradiation. The PFC response in the spleen also increases markedly from 3 to 6 weeks after whole body irradiation. By contrast, both the output of lymphocytes and the splenic PFC response remain at, or near, postirradiation levels in adult-thymectomized, irradiated mice protected from the lethal effects of haemopoietic failure by an inoculum of bone marrow cells.

This circumstantial evidence for the complicity of thoracic duct lymphocytes in haemolysin production is of the type provided by the experiments of McGregor and Gowans (1963) and Ford and Gowans (1967). These workers clearly demonstrated that (1) chronically-drained rats fail to respond in normal fashion to a primary injection of SRBC, (2) the antibody response can be elevated by infusing purified populations of small lymphocytes, and (3) the haemolysin-producing capacity of cells from the isolated and perfused rat spleen is dependent upon the number of lymphocytes in the perfusate at the time of antigenic stimulation.

Much indirect evidence is available to suggest that some thymus lymphocytes are destined to join the circulating pool (Section IA). Attempts have been made in the present series to determine whether the thymus contributes cells directly to the thoracic duct lymphocyte population and whether the majority of circulating pool lymphocytes are thymus derived. Reconstitution experiments were designed in which neonatally-thymectomized CBA mice were injected with either chromosomally-marked or semiallogeneic thymus cells from neonatal donors. The results of one experiment were clear cut. The output of

lymphocytes was increased after multiple injections of CBA/T6T6 thymus cells and almost all thoracic duct cells, stimulated into division and arrested in metaphase, carried the chromosome marker of the thymus cell donor. It might be objected that a certain number of circulating lymphocytes were contained in the thymus cell inocula from 1 to 10 day old donors and that these had replicated in host tissues. Young donors were purposely used to reduce the number of "contaminant" cells but the objection cannot be dismissed on the grounds that circulating lymphocytes could not have been contained in the thymus cell inocula. It is therefore not known with certainty whether lymphocytes produced within the thymus cortex have the potential of behaving as long-lived lymphocytes in the circulating pool .

Thymus lymphocytes differ from thoracic duct lymphocytes in that they do not elevate the output of lymphocytes when injected into draining rats (Everett, 1964; Gowans, 1964; Goldschneider and McGregor, 1968 b). Experiments have been designed in this laboratory to determine whether thymus cells must first react with antigen before they, or their progeny, recirculate (Miller and Mitchell, 1969 a). Large numbers of adult thymus lymphocytes were injected together with SRBC into lethally-irradiated syngeneic recipients which in turn received multiple injections of tritiated thymidine. The spleen cells from these mice were injected into other mice in which a thoracic duct fistula had been established. A small number of labelled small lymphocytes, in the absence of labelled large lymphocytes, was detected in smears of thoracic duct cells prepared from the draining recipients. Large pyroninophilic cells appeared in the irradiated recipients of thymus cells and SRBC and it was concluded that these cells incorporated H^3-thymidine and divided to produce a progeny of labelled small lymphocytes. This may well be so but the serious objection, based on "contaminant "

circulating lymphocytes and mentioned above, can also be raised in this instance.

Attempts to increase the size of the mobilizable pool with newborn (CBA x C57BL)F_1 hybrid thymus cells were totally unsuccessful. Nevertheless, the mice were protected from the effects of wasting disease and were tolerant of C57BL skin. They were able to reject Balb/c skin seemingly in the absence of an increased number of circulating lymphocytes. The splenomegaly and myeloid hyperplasia noticed in the thymectomized recipients were suggestive of either a GVH reaction or myelogenous leukaemia. That inoculated F_1 cells should react against CBA cells is contrary to predictions based on the genetic laws of transplantation. Studies on the "poor growth phenomenon" in irradiated F_1 mice injected with parental bone marrow cells, led Cudkowicz (1965) to propose that some C57BL antigens controlled by the H2 locus were not expressed in the cells of F_1 hybrid mice. Even if this is so, no evidence for suppression of CBA antigens in F_1 hybrid cells has been presented.

Billingham and Brent (1959) found that some A strain mice injected with (C57BL x A)F_1 hybrid lymphoid cells died unexpectedly with gross splenomegaly. Spleen cells harvested from similarly treated A strain mice did not induce GVH reactions in other F_1 mice and thus could not have been sensitized against antigens of the other parental type. These authors favoured the interpretation that the mice were affected by leukaemia. In the present system, the neonatally-thymectomized CBA mice injected from birth with newborn (CBA x C57BL)F_1 hybrid thymus cells were unable to reject C57BL skin and were thus unlikely to be sensitized against C57BL histocompatibility antigens. It is possible that a reaction to weak histocompatibility antigens determined by the H-Y locus may have contributed to the observed pathology. No attempts were made to match newborn donors and recipients with respect to sex. In addition, the operation of allogeneic inhibition (Möller and Möller, 1966) may have contributed to the

unexpected findings in the semiallogeneic "reconstitution" experiments.

A reduction in cell number was not the only defect apparent in the thoracic duct lymphocyte population following neonatal thymectomy. The population was less able to induce splenomegaly in newborn F_1 recipients and it contained a reduced number of FFC per million cells. The results of the GVH assays were similar to those reported by Rieke (Section IA) but this particular measure of immunocompetence does not provide any information on the number of cells competent to respond to foreign histocompatibility antigens. Likewise, the techniques at present in use for determining the total number of FFC in a population of lymphoid cells are incapable of achieving this aim (Section IB). However, the values obtained are useful when comparing two populations of cells for their content of FFC.

The 48 hour mobilizable pool in normal mice was found to contain in excess of 4,000 FFC whereas that in neonatally-thymectomized mice contained < 20. Six FFC were detectable in 1 million thoracic duct lymphocytes from neonatally-thymectomized mice whereas an equivalent number of lymphocytes from normal mice contained 60. It is not known whether the other 54 cells were (1) incapable of homing to the spleen of the irradiated mouse in the assay system, (2) qualitatively deficient in their ability to respond to SRBC antigens, (3) precommitted to, or engaged in responses to, other antigens, or (4) totally absent from the population. Since FFC assays are performed in irradiated mice which contain thymuses, it is unlikely that any FFC in the inoculum of lymphocytes from neonatally-thymectomized mice was unable to express itself because of the lack of a thymus humoral factor. Moreover, prior thymectomy did not influence the appearance of splenic haemolytic foci and PFC in the spleens of irradiated mice injected with SRBC and 1 million normal thoracic duct lymphocytes (Miller and Mitchell, 1968a).

The thoracic duct lymph of normal mice contained more FFC per million nucleated cells than the spleen. The role of "background" splenic PFC in the response of normal mice to SRBC has been discussed in Section IC, and the bulk of the evidence does not support the notion that they are intimately involved in the immune response. Notwithstanding this evidence, the relationship between the splenic "background" PFC and the splenic FFC remains unknown. PFC were abundant in the thoracic duct of immunized mice but none was detected in the thoracic duct lymph of unimmunized mice. The FFC in the thoracic duct are therefore not PFC or, at least, are not producing measurable amounts of 19S haemolysins.

The deficiency of FFC in neonatally-thymectomized mice does not extend to the precursors of these cells. The bone marrow of both normal and neonatally-thymectomized mice has been shown to be a source of precursors which mature into FFC in irradiated mice only under the influence of the thymus. In nonthymectomized, irradiated mice incubating bone marrow cells, a striking parallelism exists between the restoration of thymus weight (Figure 14, Kaplan and Brown, 1957; Cross, Davies, Doe and Leuchars, 1964) and the reestablishment of the splenic FFC compartment (Figure 16). This latter replenishment does not endow the radiation chimaera with the capacity to respond normally to SRBC. Restoration of the splenic PFC response is related more to the increase in circulating lymphocyte number (Figures 10 and 13). Till, McCulloch, Phillips and Siminovitch (1967) and Osoba (1968b) also showed that the PFC response in the spleen of radiation chimaeras had not returned to normal at 3 weeks after irradiation and marrow protection. With several assumptions, this data could be construed to support the contentions of Ford and Gowans (1967) that the SRBC responsiveness of the spleen is dependent upon the number of lymphocytes in the circulation rather than the resident number.

The lack of FFC precursors in the thymus does not support the hypothesis that bone marrow cells mature into FFC within the thymus environment. Nevertheless, thymic lymphopoiesis was required in the bone marrow-incubating, irradiated mouse system before FFC could be detected in the spleen. This observation led to experiments which were in essence the same as those conducted by Claman et al. (Section IC). Cells derived from the thymus and bone marrow appear to interact in the production of haemolysins in irradiated mice. Using a two host system, however, an additional finding has emerged. Thymus cells must react with the specific erythrocytes before combination with bone marrow cells and SRBC can be expected to result in significant numbers of PFC (Figure 17 and Table 6). Combinations of cells from semiallogeneic donors did not interact in this system (unpublished observation and Chaperon and Claman, 1967) and this precluded any attempt, using isoantisera, to establish whether the thymus or the marrow provided the PFC precursors.

FFC were present in thoracic duct lymphocyte inocula but not in bone marrow inocula. Bone marrow cells, however, increased the size, but not the number, of foci appearing in the spleens of irradiated mice injected with thoracic duct lymphocytes and SRBC. The peak number of PFC in irradiated mice injected with a mixture of thoracic duct lymphocytes and bone marrow cells was far greater than the sum of the PFC responses of mice injected with SRBC and either thoracic duct cells or bone marrow cells. Three interpretations of this effect in the case of the thymus-marrow interaction have appeared in the literature:

(1) The bone marrow, by promoting haemopoietic regeneration, may provide a more favourable environment for the proliferation and differentiation of PFC from their precursors in the thymus (or thoracic duct lymphocyte) inoculum. This interpretation has been favoured by Radovich et al. (1968).

(2) The thymus or bone marrow cells may repair an essential antigen-trapping or antigen-processing system in the spleen of the irradiated mouse and thereby impart a more effective stimulus to the PFC precursors in the other cell population (Miller and Mitchell, 1967b). The follicles and marginal zones of the spleen trap antigens (Nossal, Austin, Pye and Mitchell, 1966) and the lymphoid elements in these areas are destroyed by irradiation (Keuning, van der Meer, Niewenhuis and Oudendijk, 1963). Bone marrow cells (Balner and Dersjant, 1964), but not thymus cells (Bos, 1967), repopulate these areas after irradiation. One would predict therefore that the thymus cells provide PFC precursors in the thymus-marrow interaction.

(3) The other interpretation is that the bone marrow contributes PFC precursors which differentiate into PFC only in those splenic areas in which thymus (or thoracic duct) cells have lodged (Miller and Mitchell, 1967b).

Immunoserological techniques could not be used to test the various hypotheses since no synergism was obtained in the acute adoptive transfer system with combinations of semiallogeneic thoracic duct or thymus cells and bone marrow cells. Evidence for cell collaboration was obtained in irradiated/incubating mixtures of SRBC and thoracic duct and bone marrow cells from CBA and CBA/T6T6 mice donors. Of 22 scorable mitotic figures, 17 carried the chromosome marker of the bone marrow cells (Nossal, Cunningham, Mitc hell and Miller, 1968). Even though the number of cells examined was very small, the result is in line with the interpretation that the bone marrow does provide PFC precursors.

Unlike the situation in the acute restoration experiments, an interaction occurred between semiallogeneic cells in adult-thymectomized, irradiated mice injected with thoracic duct cells two weeks after bone marrow injection (Figure 22 and Table 18). In every case in which an interaction was apparent, the use of specific isoantisera unequivocally

identified the bone marrow as the major source of PFC precursors. On two occasions, (CBA x C57BL)F_1 thoracic duct cells were not particularly effective in increasing the PFC response in irradiated, adult-thymectomized, syngeneic F_1 mice protected with CBA bone marrow (Table 18). The regeneration of the spleen may have been incomplete possibly as a result of the inhibited growth of parental bone marrow in irradiated F_1 hosts (McCulloch and Till, 1963, Cudkowicz, 1965). In one of these experiments (expt. (1)), a single chimaera was injected with 100 million F_1 thoracic duct cells together with SRBC. The PFC response was increased to 65,600 PFC per spleen. The PFC were inhibited by both anti-CBA and anti-C57BL sera and were therefore of F_1 type (unpublished observation). This one mouse illustrates that the lymphocytes in the thoracic duct inocula used in expt. (1) were viable and that excellent adoptive immune responses can be obtained with huge numbers of thoracic duct lymphocytes.

The thoracic duct lymph contains many FFC and it is conceivable that these are true ARC as defined by Kennedy. In the haemolytic focus assay they may react with antigenic determinants on SRBC by producing focal accumulations of a few progeny PFC. If this is the case, it is difficult to explain why bone marrow PFC precursors should be stimulated into antibody production only in the areas of the irradiated spleen in which ARC had settled. On the other hand, the small number of PFC within each focus produced by thoracic duct cells is readily explained by postulating that two cell types are present in duct lymph. One cell responds to antigen ("reactor cell"), initiates focus formation and influences PFC precursors (bone marrow derived) in its vicinity. The thoracic duct contains an abundance of "reactor cells" but a limited number of PFC precursors. An injection of bone marrow cells supplements the number of PFC precursors in thoracic duct cell inocula, and these increase the size of each "active area" in the haemolytic focus assay after recruitment in the area of the activated "reactor cell". The

focus assay would then be a measure of the number of "reactor cells" in a population provided PFC precursors were also available to allow the expression of "reactor cells" in terms of haemolysin production.

Thymus cells and thoracic duct cells qualify for the title of "reactor cell". When injected into irradiated mice, very few mitoses were detected 3 to 7 days later but the number at 3 to 5 days after irradiation was increased substantially by a simultaneous injection of SRBC (Figure 19). More mitotic figures per 2,000 cells were detected in the spleens of irradiated mice injected with SRBC and 10 million thoracic duct cells than in mice injected with SRBC and 50 million thymus cells. The thoracic duct lymph may therefore contain more "reactor cells" per million cells. Alternatively, the progeny of stimulated thymus cells may be nonviable or may not increase, subsequent to antigenic stimulation, at the same rate as thoracic duct "reactor cells". It is clear in Figure 19 that dividing cells were still present in the spleens at 7 days after an injection of thoracic duct lymphocytes and SRBC. Very few mitotic figures were present in thymus-injected, irradiated mice at this time. This is in happy agreement with the PFC data. Thymus cells, unlike thoracic duct cells, do not give rise to PFC in the spleens of irradiated mice challenged with SRBC (Table 14). Similar experiments with bone marrow cells are meaningless since the abundance of mitotic figures in spleens undergoing haemopoietic regeneration masks any difference in the behaviour of bone marrow cells in antigen-injected and non-challenged irradiated mice.

An early burst of mitosis in thymus graft-derived cells has been described by Leuchars et al. (1964) and Davies et al. (1966) in the spleens of SRBC-challenged, adult-thymectomized, irradiated mice, injected with bone marrow cells and grafted with a chromosomally-marked thymus. A similar response of thymus graft-derived cells occurred in the regional lymph nodes of thymus-grafted radiation

chimaeras after local applications of oxazolone (Gershon, Wallis, Davies and Leuchars, 1968). Coupled with the evidence that many of the thoracic duct lymphocytes are thymus cell descendants, this data strongly suggests that the "reactor cells" in the thoracic duct are derived from the thymus.

Thymus and thoracic duct cells greatly increased the anti-SRBC PFC response in neonatally-thymectomized mice but this effect was not mediated through the simple phenomenon of direct transformation into PFC. This deduction rests heavily on the failure of specific anti-C57BL serum to inhibit PFC in allogeneically- and semiallogeneically-reconstituted neonatally-thymectomized CBA mice. Perhaps the situation is different in neonatally-thymectomized CBA mice reconstituted with CBA thymus or thoracic duct lymphocytes. Results obtained by Nossal et al. (1968) in a syngeneic CBA-CBA/T6T6 system confirm, in all respects, the present results using immunoserological techniques in an allogeneic system. Hence, the conclusions drawn from the latter system most probably apply to the syngeneically-reconstituted mouse as well. Whether they apply to the cellular events of the response to SRBC in normal CBA mice remains to be determined.

Perhaps many of the PFC in allogeneically-reconstituted mice were derived from the inoculated cells but were not expressing histocompatibility antigens of C57BL type. Celada and Klein (1967) used techniques of PFC inhibition very similar to those described here and demonstrated that all or part of both parental H2 complexes were present in heterozygous (A x A.CA)F_1 PFC. They concluded that total allelic exclusion or selective inactivation at loci concerned with the expression of H2 antigens did not occur in heterozygous immunocytes as has been proposed for codominant alleles at loci concerned with antibody synthesis (Pernis, Chiappino, Kelus and Gell, 1965). In keeping with the results of Celada and Klein, anti-CBA and anti-C57BL

sera eliminated, or at least inhibited antibody secretion from, (CBA x C57BL)F_1 PFC (Table 8). Do these findings necessarily apply to F_1 cells in the presence of CBA cells? It is apparent in Table 15 that F_1 thoracic duct cells, when injected into heavily-irradiated mice gave rise to a small number of PFC which were inhibited by both antisera. F_1 cells can therefore be detected in the environment of an abundance of CBA cells. In view of this demonstration, one cannot seriously entertain the proposal that F_1 cells had lost part of the H2 complex in the neonatally-thymectomized CBA mouse. Such a proposal must infer a complete loss of all H2 complexes in those experiments in which C57BL lymphoid cells enhanced the PFC response in neonatally-thymectomized CBA hosts. Complete suppression of antigens of the type determined by the H2 locus has not been recorded (Hellström and Möller, 1965).

The inoculated F_1 and allogeneic lymphoid cells may have induced a host-versus-graft reaction, which in the presence of SRBC, enabled host precursors to proliferate and mature into PFC. However, neonatally-thymectomized CBA mice were unable to reject C57BL skin grafts and irradiated F_1 thoracic duct cells did not restore the PFC response to SRBC. Furthermore, reconstitution was effected with syngeneic inocula in which case no histo-immunological stimulus would have been experienced by the host cells.

Since host cells were responsible for antibody production in reconstituted thymectomized mice, it becomes important to test other inocula for their capacity to influence the responsiveness of the host. Non-cellular inocula have been shown to partially abrogate the immunosuppressive effects of x-irradiation presumably by means of promoting recovery in, or affording protection to, host cells (Jaroslow and Taliaferro, 1956; Jaroslow, 1960). Bergland (1965) and Braun and Nakano (1965) also reported an enhancing effect of oligodeoxyribonucleotides from thymus DNA, spleen homogenates, and nuclei of thymus cells, in

the haemolysin response of rats and mice to SRBC. Large numbers of heavily-irradiated thymus and thoracic duct cells failed to elevate the peak number of PFC in neonatally-thymectomized mice injected with SRBC (Table 10). Similar results have been reported using irradiated thymus cells in the thymus-marrow interaction (Claman et al., 1966b; Cheng and Trentin, 1967b). Yeast cells, thymus extracts, and bone marrow cells were also ineffective in the present system.

The controls suggest that the response of thymectomized mice cannot be increased simply by injecting large numbers of cells and that intact and viable lymphoid cells, capable of cell division, are required for successful reconstitution. This latter point derives from the ascertion that "...the essential lesion produced by doses of X-rays in the range of 500 - 2000 r is an impairment in the capacity for cell division" (Kaplan, 1966). It remains an open question, however, whether the extreme radiation sensitivity of lymphocytes is a reflection of a direct effect of X-rays or whether it is a manifestation of cell death at mitosis.

Human and guinea pig thymus cells were totally inactive in the reconstitution experiments and yet allogeneic thymus cells were most efficacious. Might not thymus cells from xenogeneic donors, not too distantly removed from the mouse in a genetic sense, result in a moderate number of PFC? As seen in Table 10, 100 million rat thymus cells significantly increased the PFC response in neonatally-thymectomized CBA mice, albeit slightly. Anti-CBA serum inhibited the PFC in the spleens (unpublished observation) but no specific antirat serum was available to confirm that the PFC were not derived from rat thymus cells.

The most satisfactory control preparation in this and related systems would seem to be an inoculum of cells from tolerant donors. In the experiments of Gowans and his colleagues (Section IB), thoracic duct small lymphocytes increased the anti-SRBC haemolysin response

in lethally- and sublethally-irradiated rats. Lymphocytes from fully tolerant donors were totally ineffective and were indeed less active than an injection of yeast extract in sublethally-irradiated rats (McGregor et al., 1967). This finding provides cogent proof that the response of the irradiated host is not solely responsible for antibody production in lymphocyte-injected rats and, by inference, the lymphocytes from normal rats must be immunologically active. Tolerance in the rats was induced with multiple injections of SRBC, the injections commencing early in life. This method of tolerance induction is unsatisfactory in CBA mice (Miller, J.F.A.P., personal communication).

Dietrich and Dukor (1967), Aisenberg (1967) and Aisenberg and Davis (1968) demonstrated that mice injected with both SRBC and cyclophosphamide failed to respond, by producing specific antibodies, when subsequently injected with SRBC. Control mice, injected with cyclophosphamide only, recovered rapidly from the nonspecific drug-induced suppression of immunological responsiveness. This method of tolerance induction was employed in experiments addressed to the question of whether lymphoid inocula increase the PFC response in neonatally-thymectomized mice in a nonspecific manner.

The results of the reconstitution experiments using "tolerant lymphocytes" (Tables 11 and 12) may be summarized as follows:-

(1) The difference in the SRBC responsiveness of tolerant and nontolerant mice was particularly striking.

(2) Tolerance was quite specific in that both SRBC-tolerant and nontolerant mice responded equally well to horse erythrocytes (HRBC).

(3) Thoracic duct cells from SRBC-tolerant mice were as effective as cells from nontolerant mice in adoptively transferring HRBC responsiveness to neonatally-thymectomized mice but were far less effective in adoptively transferring SRBC responsiveness.

(4) Thoracic duct cells from SRBC-tolerant mice were effective in significantly elevating the response of neonatally-thymectomized mice to SRBC.

The latter finding requires an explanation. Tolerance in the donors was by no means complete (c.f. McGregor et al., 1967) and an unpublished observation is pertinent to the present discussion. On one occasion, six mice treated with SRBC-cyclo were cannulated, the lymphocytes pooled and injected into neonatally-thymectomized mice together with SRBC. The PFC responses in the recipients were markedly elevated and no thymus remnants were evident macroscopically. When the 6 donors were challenged with SRBC, one was found to have a normal splenic PFC response. The inoculum had been contaminated by thoracic duct lymphocytes from a single nontolerant mouse and the small number of cells was sufficient to effect a response of considerable magnitude in the thymectomized recipients. In no experiment was a difference recorded in the number of lymphocytes emerging from the thoracic duct of tolerant and nontolerant mice. The results suggest that only a few "nontolerant lymphocytes", in the presence of many "tolerant lymphocytes", are required in the inoculum in order to increase the SRBC responsiveness of neonatally-thymectomized mice.

The failure of normal thoracic duct and spleen cells to effectively break tolerance and increase the response of tolerant mice to normal levels might, at first sight, tend not to support the aforementioned suggestion. "Nontolerant lymphocytes" would presumably be harboured in company with "tolerant lymphocytes" in the lymphoid tissues. It is not known, however, whether the inoculated spleen or thoracic duct cells homed to the spleen of the tolerant hosts there to be triggered by antigen. The tolerant mice, unlike neonatally-thymectomized mice, are not deficient in thoracic duct lymphocytes and the injected cells may have been unable to penetrate to sites where antigenic stimulation is presumed to occur.

It was not anticipated that 40 million spleen and thoracic duct-marrow cells would behave so erratically in tolerant hosts. Such inocula could be expected to contain large numbers of both "reactor cells" and PFC precursors. 7S antibodies have been shown to inhibit the 19S PFC response to SRBC (Henry and Jerne, 1968; Uhr and Möller, 1968). No haemagglutinating antibodies were detected in the sera of 10 SRBC-cyclo treated mice prior to injections of spleen cells and SRBC (unpublished observation).

The numerous unknown factors do not encourage an exhaustive discussion on the nature of the lesion of tolerance in SRBC-cyclo treated mice. There is, however, some evidence in this model that the thoracic duct lymphocytes may not be the only cells affected by tolerance induction. No information is available concerning the operation of extracellular mechanisms (Eisen and Karush, 1964; Hemphill, Segre and Myers, 1966) and the involvement of macrophages (e.g. Nachtigal, Greenberg and Feldman, 1968) in the maintenance of tolerance in this system. Interestingly, there is one report that normal rat thoracic duct cells are unable to break tolerance to SRBC in situations where mixtures of thoracic duct lymphocytes and peritoneal macrophages are quite successful (McCullagh, 1968). Moreover, Gallily and Feldman (1967) and Feldman and Gallily (1967) have clearly demonstrated that macrophages are involved in the adoptive antibody response to Shigella antigens in heavily-irradiated mice.

It might be suggested that inducing a response to HRBC would elevate the SRBC responsiveness of neonatally-thymectomized mice injected with lymphocytes from SRBC-tolerant mice. Four experiments were performed to test this hypothesis. The mean SRBC-PFC response of 17 neonatally-thymectomized mice injected with "SRBC-tolerant lymphocytes" and both SRBC and HRBC was increased above that in recipients challenged with SRBC only. The increase, however, was not significant. Moreover in the SRBC-tolerant mice, an injection of both SRBC and HRBC did not break tolerance to SRBC even though a

normal PFC response to HRBC occurred. Control mice, treated with cyclo only, produced fewer PFC to SRBC when injected simultaneously with HRBC. The phenomenon of antigenic competition may have contributed to this observation as has been inferred from the comparable effect of injecting SRBC and HRBC into normal mice (Radovich and Talmage, 1967).

Thymus cells were as effective as thoracic duct cells in elevating the PFC response to SRBC in neonatally-thymectomized mice. This finding contrasts with the results of many previous studies on the immunological activity of thymus cells (Section IC). In addition, large numbers of thymus cells were unable to significantly increase the responsiveness of thymectomized radiation chimaeras challenged with SRBC two weeks after irradiation and marrow protection. Thoracic duct lymphocytes were most effective in such hosts. It is highly improbable that the presence of "contaminant" circulating lymphocytes accounted for the effects observed with 10 and 50 million thymus cells in neonatally-thymectomized mice. Thus 1.5 million thoracic duct cells (equivalent to a contamination level of 15%) produced by no means as great an effect as 10 million thymus cells.

The spleens of 3 to 5 weeks old neonatally-thymectomized mice contained more nucleated cells than those of the radiation chimaeras and the spleens were visibly larger. The difference in spleen size was particularly noticeable at the time the mice were killed for the PFC assays. Since thymus cells home to the spleen in large numbers (Section IA), the enlarged spleen may trap and recruit more of the injected thymus cells. Thus contention is not supported by the results of more recent experiments. Cr^{51}-labelled thymus cells were injected into groups of neonatally-thymectomized mice and thymectomized radiation chimaeras. The average percentage of recoverable radioactivity

in the spleens of neonatally-thymectomized mice at 4 and 24 hours after thymus cell injection was not increased above that in the spleens of the chimaeras.

One suggestion which would resolve this dilemma is that thoracic duct cell inocula differ from thymus cell inocula in that they contain cells which repair a radiosensitive and essential antigen-processing system in irradiated mice. This cell system would presumably be present in the spleens of neonatally thymectomized mice. With the assumption that the antigen-processing cell compartment is thymus independent, one would expect regeneration of this system to be complete in thymectomized radiation chimaeras at 6 weeks after irradiation and marrow protection. This time point coincides with the complete recovery of PFC responsiveness in nonthymectomized radiation chimaeras. At both 4 and 6 weeks after irradiation, however, thymus cells were inferior to thoracic duct lymphocytes in adoptively transferring SRBC responsiveness (Table 17). The underlying reason for the disparity of thymus cell behaviour in the two host types must remain unknown but will be raised again later in this discussion.

The statement " ... thymus cells were as effective as thoracic duct cells . . . ." applies only to the peak PFC response in reconstituted neonatally-thymectomized mice. It is evident in Figure 18 that the PFC number remained elevated in mice given thoracic duct cells at a time when the number in thymus-injected mice had fallen. This may reflect either a longer life span of PFC or continued recruitment of "reactor cells" or PFC precursors in the spleens of thoracic duct cell recipients. Rat thymus and thoracic duct lymphocytes differ in their capacity to recirculate and in the present system, the thoracic duct cells may have been recruited into the spleen, to initiate antibody production in host cells, for a longer period of time after antigen challenge. At variance with this conclusion is the finding that effective antigenic stimulation, in irradiated rats at least, is much reduced after

the first 24 hours following SRBC injection (Ford, 1968). 7S PFC analyses at later time points after thymus and thoracic duct cell injections into neonatally-thymectomized mice may be particularly illuminating. The elevated number of 19S PFC may be a result of a lack of inhibitory 7S antibody in the serum or, less conceivably, a failure of 19S PFC to convert to 7S PFC.

The results of several recent experiments have been interpreted in terms of cellular interactions in haemolysin production. A "premium effect" of large cell inocula in irradiated recipients was described by Celada (1967) in the adoptive secondary response to human serum albumin. Gregory and Lajtha (1968) observed a similar phenomenon using both the PFC assay and the haemolytic focus assay in the adoptive immune response to SRBC in irradiated mice. The number of haemolytic foci in the spleens of irradiated recipients of spleen cells and SRBC was linearly related to the number of spleen cells injected, the line of best fit having a slope of one. By contrast, the peak PFC response, measured over a higher cell dose range, increased allometrically with graft size. Of much interest is the statement in discussion that bone marrow cells produced a low but increasing number of PFC with increasing cell dose. No evidence of a 2nd order interaction was reported to have been obtained with bone marrow cells. These authors concluded that the production of PFC from their precursors in the spleen could be explained in terms of a cellular cooperative process.

Mosier and Coppleson (1968) have presented data which they believe is suggestive of a three cell interaction in the primary immune response to SRBC in vitro. By using various numbers of fractionated and unfractionated spleen cells, they were able to construct log cell dose - log PFC response plots and measure the slope of the regression line. In cultures of spleen cells the slope of the line approached 3.0. Taking advantage of the property of differential cell adherence to glass, two fractions of spleen cells were prepared. Various doses of non-adherent cells were added to cultures containing a fixed number of

adherent cells and the slope of the dose-response regression line was found to approximate 2.0. Mosier and Coppleson concluded that 2, if not 3, cells were interacting in the in vitro immune response to SRBC. One of these cell types was probably a macrophage. The data published indicates that the number of PFC per culture was <100 in most cases and thus much less than the responses obtained by Mishell and Dutton (1967) in an identical in vitro system. The conclusions of Mosier and Coppleson are therefore based on very few PFC obtained in a system which is capable of promoting much higher PFC responses in isolated spleen cells. Furthermore, a time course study was not performed and only the 4 day PFC response was examined.

The interpretations of these two sets of data, as well as those of the putative thoracic duct-marrow and thymus-marrow cell interactions, may be altered by the recent work of Henry and Jerne (1968). Purified 19S haemolysins, with immunological specificity rather than species specificity, have been shown to enhance the PFC response at certain concentrations. A positive feedback influence of 19S antibody may accelerate the generation of PFC from their precursors or effect the recruitment of more "reactor cells" or PFC precursors. As this effect is a dose dependent phenomenon, a threshold amount of 19S antibody (secreted by a certain number of PFC) may be required before increased numbers of PFC are produced (vide infra).

What then is the nature of the interaction between thymus or thoracic duct lymphocytes (a number of which are probably thymus cell descendants) and bone marrow-derived cells in the production of 19S haemolysins in reconstituted CBA mice ? A number of possibilities will be discussed in succeeding paragraphs.

Perhaps the interaction is more apparent than real, the lymphocytes acting as trephocytes (Section IA) which simply provide nutrient or precursor substances to the cells of the bone marrow cell line. Experimental results militating against the validity of this conclusion

are (1) the negligible reconstitutive activity of irradiated thymus and thoracic duct cells, bone marrow cells, thymus extracts, yeast cells and xenogeneic thymus cells, and (2) the reduced efficacy of lymphocytes from specifically tolerant donors in the reconstituted neonatally-thymectomized mouse system.

Perhaps antigen-stimulated,thymus-derived cells manufacture and release substances which influence the differentiation of bone marrow-derived cells. The embryonic pancreas contains two separable populations of cells; one is peripherally located in explants and incorporates tritiated thymidine. This proliferating population is essential for differentiation of cells, at the centre of the explant, into zymogen-producing cells (Bernfield and Fell, 1967). The nature of the influence of one cell type on the other remains to be determined. Thymus-derived, proliferating cells may elaborate nonspecific pharmacologically-active substances (Burnet, 1968a, b) akin to those associated with the various and diverse manifestations of delayed hypersensitivity reactions in vivo and in vitro (Humphrey, 1967, Lawrence, 1967).

Several years ago, Dutton and Harris (1963) produced evidence that substances liberated by irradiated or nonirradiated spleen cells from primed rabbits, exposed momentarily to antigen in vitro, stimulated DNA synthesis in nonirradiated unprimed spleen cells. Simple saline extracts of thymus do not substitute for thymus cells in the reconstituted thymectomized mouse system or the thymus-marrow system of Claman et al. (1968). Nevertheless, the possibility must be seriously entertained that proliferating, antigen-activated, thymus-derived lymphocytes produce nonspecific substances with adjuvant-like properties. There are two findings in the present series which are relevant to this point. Firstly, in the two host system, in which thymus cells were stimulated with heterologous erythrocytes and the spleens of the mice transferred to secondary irradiated hosts together with bone marrow cells, the homologous erythrocytes had to be given to the first host before a significant response occurred to that erythrocyte type in the

second host (Figure 17 and Table 6). Secondly, and more cogently, a simultaneous injection of SRBC and HRBC did not significantly elevate the PFC response in neonatally-thymectomized mice injected with "tolerant lymphocytes" above that obtained with SRBC only. Numerous HRBC "reactor cells" could be expected to be present in the inoculum since the anti-HRBC PFC response in the hosts was markedly increased. Any factor elaborated by thymus-derived cells responding to HRBC should have been available to nonspecifically induce anti-SRBC PFC production in bone marrow-derived cells.

Perhaps thymus-derived lymphocytes elaborate specific inducer factors after interaction with erythrocyte antigens. The possible candidates include RNA (Fishman and Adler, 1963; Gottlieb, Glisin and Doty, 1967; Mosier and Cohen, 1968), RNA-antigen complexes (Askonas and Rhodes, 1965; Friedman, Stavitsky and Solomon, 1965; Fishman and Adler, 1967; Pinchuck, Fishman, Adler and Maurer, 1968), and far more speculatively, episomic genetic elements (Jacob, 1960) or "viral antibodies" (Smithies, 1965). The RNA-antigen complex may have inducer properties or the RNA may behave as an adjuvant, the complex then acting as a "superantigen". The macrophage has been implicated in the processing or presentation of antigen and in the production of informational low molecular weight RNA. The above speculation may therefore be extended to a consideration of whether thymus-derived lymphocytes have macrophage-like properties.

On the basis of the observed histological consequences of cortisol administration and the patterns of H^3 thymidine incorporation into lymphocytes in various anatomical localities, Craddock, Winkelstein, Matsuyuki and Lawrence (1967a, b) have proposed that thymus and marrow lymphocytes are short-lived macrophage precursors. Many years earlier, Trowell (1958) had suggested that lymph-borne lymphocytes were "pocket macrophages" and many morphologists have long contested that lymphocytes transform into macrophages in

sites of inflammation (e. g. Rebuck, 1947). Autoradiographic studies have established the bone marrow as the primary source of macrophage precursors (Spector, Walters and Willoughby, 1965; Volkman and Gowans, 1965a, b; Volkman, 1966; van Furth and Cohn, 1968). Virolainen (1968) used a culture system which preferentially stimulated the growth and proliferation of macrophages in vitro and was unable to demonstrate macrophage precursors in the thymus or lymph nodes. The donors of the cultured cells were syngeneic radiation chimaeras injected with chromosomally-marked haemopoietic cells and lymphoid cells from various sources.

In radiation chimaeras subjected to intensive stimulation of the reticuloendothelial system, and in the GVH reaction, some dividing liver macrophages may be derived from thoracic duct lymphocyte inocula (Boak, Christie, Ford and Howard, 1968). It would seem, therefore, that cells of lymphocyte morphology may under certain circumstances behave as macrophage precursors. The conclusion that macrophages derive from long-lived recirculating small lymphocytes cannot be justified of the basis of the findings of the Edinburgh group. The thoracic duct lymph of rats and mice may contain a variety of morphological cell types (e. g. Braunsteiner, Höfer and Sailer, 1961; Marchesi and Gowans, 1964; Morse and Riester, 1967), some of which may be phagocytic (Spector, W. G., personal communication). Thymus lymphocytes have not been shown to transform into macrophages and neonatally-thymectomized rats and mice are certainly not lacking in macrophages (Miller and Howard, 1964; Corsi and Giusti, 1967b). Nevertheless, the possibility cannot be discounted that a subclass of macrophages is absent in thymectomized animals.

Bone marrow cells do not respond, by producing PFC, when injected together with large numbers of SRBC into irradiated mice (Table 14; Claman et al., 1966a, b; Shearer, Cudkowicz, Connell and

Priore, 1968). Likewise, neonatally thymectomized CBA mice do not produce more PFC to 10^9 SRBC than to 10^8 SRBC (Table 12). Hence, large antigen doses do not obviate the requirement for thymus or thoracic duct lymphocytes in these two systems. The opposite conclusion was reached in the recent study of Sinclair and Elliott (1968b) in which neonatally-thymectomized, sham-operated, and normal Swiss mice were challenged with 10^4 to10^9 SRBC. The peak titres and kinetics of 7S and 19S haemolysin production in young neonatally-thymectomized mice injected with 10^9 SRBC were similar to those in sham-operated mice injected with 10^7 SRBC. Neonatally-thymectomized Swiss mice were essentially unable to respond to 10^7 SRBC by producing antibodies.

Direct comparisons between the results of Sinclair and Elliott and those presented here are not strictly possible since nothing is known of the cellular events of PFC production, or the number of PFC, in Swiss mice injected with SRBC. It is possible that neonatally-thymectomized Swiss mice do not have as severe a deficiency in "reactor cells" as do their CBA counterparts. Increasing the dose of SRBC may effect the triggering of more of these cells and thus increase the opportunity of collaboration between "reactor cells" and PFC precursors. The 1,000 to 3,000 PFC in the spleen of the neonatally-thymectomized CBA mouse may reflect maximal recruitment of the few available "reactor cells". The results were quite clear cut in the case of the acute adoptive response to SRBC in irradiated mice injected with bone marrow cells. This cell population contains PFC precursors yet enormous numbers of SRBC did not stimulate any differentiation into PFC. If the PFC precursors in the bone marrow-derived cell population have no antigenic reactivity and have an essentially passive role to play in the interaction with thymus-derived cells, then the latter must be the more likely targets for tolerance induction and the more likely candidate for the carriage of specific immunological memory.

It has been shown that the phenomena of tolerance and immunological memory are associated with the thoracic duct lymphocyte popu-

lation in some antigenic systems (McGregor et al., 1967; Gowans and Uhr, 1966; Table 12). Smith, Isaković and Waksman (1966) were able to demonstrate an accentuated delay in the development of BSA responsiveness in adult-thymectomized, irradiated rats injected with bone marrow and grafted with thymus from BSA-tolerant rats. Similar results using BγG were obtained by injecting the shielded thymus of irradiated rats with the specific antigen, but not by injecting BγG intraperitoneally or intrasplenically (Staples, Gery and Waksman, 1966; Horiuchi and Waksman, 1968). It is not known whether tolerance was induced in the thymus lymphocyte population which was destined to peripheralise, or whether "low zone" tolerogenic levels of antigen were liberated from the thymus which temporarily induced tolerance in the regenerating population of peripheral cells.

Further experiments suggest that thymus graft-derived cells can be made unresponsive by antigen treatment. Gershon et al. (1968) used adult-thymectomized, irradiated, marrow protected mice implanted with a chromosomally-marked thymus graft and induced varying degrees of unresponsiveness to SRBC by multiple antigen injections during the immediate postirradiation period. They recorded a reduced mitotic response of thymus-graft derived cells in the spleen and lymph nodes after challenge with either SRBC or human RBC but a normal mitotic burst after oxazolone. The failure to elicit a mitotic response to human RBC does not indicate that the thymus graft-derived cells had been nonspecifically inactivated. Gershon et al. did not report data on the antibody response to human RBC and it is therefore not known whether cross tolerance to human RBC had been induced by the repeated injections of SRBC.

Results pertaining to the susceptibility of thymus cells to tolerance induction have recently appeared. Taylor (1968) has reported that thymus and marrow cells interact in the immune response to BSA in irradiated mice. Thymus cells taken from mice injected with BSA

24 hours previously did not collaborate with normal bone marrow in the production of antibodies. By contrast, antigen administration to the donor mice did not affect the ability of marrow cells to interact with normal thymus cells. No data has been presented, however, and specificity control have not been included. It would be of interest to know whether thymus cells from mice injected with other heterologous serum proteins are able to collaborate with bone marrow cells in the production of anti-BSA antibodies. In the present series, thymus cells taken from mice challenged 24 hours previously with either SRBC or HRBC were quite capable of elevating the PFC response to SRBC in neonatally-thymectomized mice (unpublished observation). If BSA does induce specific immunological tolerance in the thymus lymphocyte population, then the difference in the two results may be related to differences in the penetrability of antigens into the thymus.

Despite the apparent negative SRBC reactivity of bone marrow cells mentioned above, it is likely that the bone marrow-derived cell is competent to respond to antigenic determinants. This conclusion stems from the numerous demonstrations that the absence of the thymus does not affect the humoral antibody response to many antigens. In view of the complexity of the SRBC "antigen" it might be anticipated that, after parenteral injection, many antigenic determinants are presented to the reactive cells with or without the intervention of macrophages. Studies with chemically-defined hapten-carrier complexes in delayed sensitivity reactions and antibody production have indicated that the recognition of two or more antigenic determinants is involved, perhaps simultaneously, in the elicitation of a measurable response to a single determinant. Tolerance or genetically-based unresponsiveness to the protein or oligopeptide carrier will inhibit a response to the hapten. The elicitation of secondary responses in vivo, or in lymphoid populations in vitro, requires the presence of the same carrier as was used in the primary stimulus. The "carrier

effect" is not absolute, however, and by increasing the concentration of hapten conjugated to nonhomologous carriers, the so-called "carrier specificity" may be overcome (e.g. Mitchison, 1967; Rajewsky and Rottlander, 1967; Benacerraf, Green and Paul, 1967).

As might be expected (Nossal, 1962), individual antibody-forming cells do not produce antibodies against both carrier and hapten (Green, Vassalli, Nussenzweig and Benacerraf, 1967). This finding, and the results of Mitchison (1967), favour the notion that antigenic determinants in hapten-carrier conjugates are not regionally associated in the sense that recognition of the carrier need not necessarily involve a region of the protein molecule adjacent to the hapten. The diagrams of Levine (1965), do infer that the local environment of the hapten is all important in the expression of the "carrier effect". The one cell - one basic receptor - one antibody dogma in antibody production insists that one must invoke the participation of at least two different cells in the recognition of the haptenic determinant and the carrier determinants. Mitchison tentatively suggested that the macrophage was the cell type responsible for the absorption, if not the recognition, of carrier protein, and hence the presentation of determinants to target hapten-reactive cells. In the system of Schierman and McBride (1967), chicken erythrocyte A_2 isoantigens were nonimmunogenic but when coupled with B_3 antigens, on the same erythrocytes, anti-A_2 antibodies were produced. This finding suggests that a "carrier effect" may be involved in the immune response to determinants on foreign erythrocytes.

The complicity of thymus-derived lymphocytes in the mediation of the "carrier effect" must now be considered. All the evidence in the present series of experiments is in accord with the hypothesis that thymus-derived lymphocytes respond to antigenic determinants on SRBC other than those directly involved with haemolysin production. Thymus-derived cells may therefore be carrier-reactive cells. The only satisfactory hypothesis which relates the excessive lymphopoietic activity of the thymus to the evidence that many lymphocytes die within the organ,

is that cells with restricted reactivity patterns are generated and screened within the thymus (Section IC). The "carrier effect" hypothesis in the present system must therefore ascribe unispecific reactivity to both thymus-derived and bone marrow-derived cells and these two cell types must come into close association within lymphoid organs.

Bretscher and Cohn (1968) have pointed out that it is difficult to conceive of the mechanism whereby two rare cells are focussed into close proximity in the presence of antigen. This as a conceptual difficulty must not go unchallenged. Young and Friedman (1966) demonstrated that early 19S PFC were associated with the white pulp in the spleen and suggested that they originated in follicles and germinal centres. If this is so then it tends to support the contentions of Bretscher and Cohn. Large-scale migration of circulating lymphocytes through lymphoid follicles does not occur (Gowans and Knight, 1964; Austin, 1968) and these antigen-containing sites (e.g. Nossal, Ada and Austin, 1964) would not accommodate or promote a sufficient number of contacts between rare cells. More recently, Fitch and Rowley (1967) and Fitch, Stejskal and Rowley (1969) have demonstrated that PFC are not associated with lymphoid follicles and germinal centres and that PFC first appear in the marginal zones and periarteriolar lymphocyte sheaths. Germinal centres and lymphoid follicles may harbour haemolysins later in the response since in the papers of Young and Friedman (1966) and Bergland, Markey and Mergenhagen (1967) many haemolytic foci were present in the spleen at a time when 19S PFC were almost undetectable.

The periarteriolar lymphocyte sheaths and marginal zones are sites of extensive cell migration (Section IA). Furthermore, the early burst of mitosis in thymus-derived cells may increase the number of specific carrier-reactive cells in the system. Both mechanisms would then provide the means of increasing the probability of contact between

the postulated carrier-reactive cell and the hapten-reactive cell. Moreover, it is likely that many of the multitude of antigenic determinants on heterologous erythrocytes behave as carrier molecules. Hence, considerable numbers of clonally-individuated, thymus-derived, carrier-reactive cells may be involved in the presentation of haptenic determinants to bone marrow-derived PFC precursors.

Bretscher and Cohn have retained the attractive idea that both carrier-reactive and hapten-reactive cells are unispecific in their antigenic reactivity. To overcome what they consider is the unlikely event of two rare cells coming into close anatomical association, these authors have proposed that certain cells passively absorb (preformed ?) carrier antibody molecules. These molecules by combining with the carrier portion of the antigenic molecule, present the hapten-reactive cell with exposed and suitably-oriented haptenic determinants. They further postulate that the presentation of the hapten is such that receptors on the hapten-reactive cell are stretched. This physical disturbance at the cell surface presumably constitutes hapten recognition and the initial step in the process of cellular activation which ultimately results in antibody production.

The extended hypothesis of Bretscher and Cohn might infer that thymus-derived cells passively absorb carrier antibodies. This then clearly warrants a closer look at the report of Henry and Jerne (1968). As mentioned previously, the work of this group indicates that immunologically-specific, 19S haemolysin-containing preparations have positive feedback properties in haemolysin production. Might not the purified macroglobulin preparation of Henry and Jerne contain carrier antibodies ? Alternatively, is it not conceivable that thymus-derived cells absorb 19S haemolysin molecules themselves which, in combination with the specific antigenic determinants and the carrier receptors on the thymus-derived cell, present, distort, or expose other hidden determinants to the target bone marrow-derived PFC

precursors? Henry and Jerne considered this possibility but adjudged the property of antibody absorption to be within the gamut of macrophage activity.

It is equally conceivable that the enhancing effect of 19S haemolysins in PFC production is simply to promote disintegration (e. g. Litt, 1967) or opsonization of foreign erythrocytes (e. g. Jenkin and Rowley, 1961). The uptake of erythrocyte antigenic material into macrophages may be a necessary prerequisite for the induction of the cellular events of haemolysin production (Ford et al., 1966). If this is the only function of 19S haemolysins in the system of Henry and Jerne, it might be anticipated that the increased number of PFC should be detected early in the response to SRBC. This does not seem to be the case and increased numbers of PFC are only apparent at the peak of the response. Perhaps another cell type (e. g. thymus-derived cell) is required in sufficient numbers before 19S haemolysins become operative in promoting PFC production.

One interesting feature of the efficacy of thymus lymphocytes in the present study, is that the cells are only effective in hosts which are themselves capable of mounting a significant PFC response to SRBC. Mice treated with antilymphocyte serum (Martin and Miller, 1968a), neonatally thymectomized mice, and well-established thymectomized radiation chimaeras, produce PFC in the spleen after a primary injection of SRBC and thymus cells further increase this response. By contrast, newly-established thymectomized radiation chimaeras do not respond to SRBC by producing PFC and thymus cells, unlike thoracic duct cells, are virtually without effect in the adoptive transfer of SRBC responsiveness. A certain titre of early 19S haemolysins (and thus a certain number of PFC) may be required in the system before significant levels are absorbed onto thymus-derived "reactor cells". In this situation the passively absorbed antibody may be in a position to expose or focus antigenic determinants onto other PFC precursors.

Thoracic duct lymphocyte inocula are able to produce PFC in the spleens of irradiated mice. The PFC precursors in the thoracic duct lymph may be differentiated to the extent that they do not require the influence of "reactor cells" to produce progeny PFC. Thoracic duct lymphocyte-derived PFC in thymectomized radiation chimaeras cannot be detected in the presence of abundant numbers of bone marrow-derived PFC. Nevertheless, thoracic duct cell inocula, unlike thymus cell inocula, may provide the necessary amount of early 19S haemolysin to cascade the response mediated by "reactor cells" and bone marrow-derived PFC precursors.

There is one interpretation of the thymus-marrow cell interaction which does not invoke information transfer between cells and which embraces the following facts and suggestions:

(1) 19S haemolysins are required before it is possible to achieve the full expression of the interaction between thymus-derived "reactor cells" and bone marrow-derived PFC precursors.

(2) A "carrier effect" is involved in the immune response to erythrocyte antigens.

(3) The probability of contact between unispecific reactive cells is slight.

(4) Specific tolerance can be induced in thymus or thymus-derived "reactor cells".

(5) The thymus is more intimately concerned with cell-mediated immunities.

(6) There is no evidence whatsoever that thymus or thymus-derived cells secrete any known intact immunoglobulin molecule with antibody activity.

This hypothesis is that thymus-derived cells are carrier-reactive cells in their own right and bear recognition units on their surface which are an expression of gene action within the cell. In the latent period of

the haemolysin response, some highly differentiated bone marrow-derived PFC precursors in the spleen and/or the circulating pool respond to the appropriate determinants and produce a small number of PFC. Alternatively, a "carrier effect" may be responsible for the production of relatively few PFC in the spleen in this period. During the exponential phase of the response, activated thymus-derived cells passively absorb early 19S haemolysins and, following combination with antigen molecules, focus antigenic determinants onto other PFC precursors.

If point (3) above is invalid, and if the positive feedback influence of 19S haemolysins is mediated by antigen-processing macrophages, it is most likely that a "carrier effect" is solely responsible for haemolysin production in combinations of thymus-derived and bone marrow-derived cells. The latter hypothesis has merits of simplicity and is all the more credible if many determinants on foreign erythrocytes can serve as carrier molecules.

There is one report which has relevance to point (6) mentioned above. Chou, Dubiski and Cinader (1967) increased the immunogenicity of human serum albumin (HSA) in rabbits by incubating the antigen with allogeneic red cells or viable nucleated lymphoid cells including thymus cells. By using donors and recipients which differed in the allotypic specificity of their immunoglobulins, Chou et al. demonstrated that only recipient-type anti-HSA antibodies were present in the sera at various times after neonatal injection of the cell-associated antigen. In contrast to the situation in recipients of lymph node cells, few thymus cell recipients contained donor type immunoglobulins in the sera at 14 days of age. Nevertheless, a largernumber of thymus cell recipients contained a higher concentration of donor-type immunoglobulin throughout the 160 day observation period. The authors porposed that thymus cells, by virtue of their relative immaturity, possessed a greater potential for establishing cellular chimaerism and thus for producing immunoglobulins whilst resident in the tissues of the recipient.

Provided that the donor cells had not transferred specific information for the expression of allotypy to recipient cells, the data suggests that thymus-derived cells have the potential for synthesizing circulating immunoglobulins. This is contrary to the implications of the hypothesis developed above. It is possible that a certain proportion of thymus cells have the ability to dedifferentiate into "stem cells" of the type which are thought to be sequestered into the thymus from the circulation (reviewed in Miller and Osoba, 1967). Alternatively, thymuses removed at any one particular time may contain a number of immigrant "stem cells" which have yet to come under the influence of the humoral factors elaborated by the epithelial cytoreticulum (Section IC). The "thymus-repopulating unit", which presumably originates in the bone marrow, could retain the potential for immunoglobulin synthesis prior to undergoing proliferation and differentiation within the thymus environment. Bone marrow cell inocula were not included in the experiments of Chou et al. and it would be of interest to know whether such inocula promote the appearance of large amounts of persisting donor-type immunoglobulins in a large proportion of the newborn recipients.

The answers to two questions are required before a clear understanding of cellular interactions in haemolysin production will be possible. These questions are relevant to any proposal based on the transfer of information between thymus-derived cells and bone marrow-derived cells. Can tolerance to SRBC be induced in the bone marrow cell line and does the product of the bone marrow-derived PFC carry any marker distinctive for the thymus-derived cell? An additional question may be posed. If the majority of thoracic duct lymphocytes are thymus derived and if immunological memory is carried by populations of thoracic duct cells, what will be the result of combinations of primed thoracic duct lymphocytes and virgin bone marrow-derived cells? If the avidity of the antibody molecules

elaborated by cells of the bone-marrow inoculum is increased, then this must surely be good evidence for a transfer of information between the two cell types. The answers to such questions must await the incorporation of simpler antigens or hapten-carrier conjugates into the present system. The antigens must of necessity require the presence of thymus-derived cells to elicit an antibody response; that is the antibody responses must be "thymus dependent".

Experimental and clinical evidence suggests that cell-mediated immunities, such as delayed-type hypersensitivity and the homograft reaction, are severely affected by the absence of the thymus. Furthermore, a strong case can be made out for the concept that recirculating lymphocytes, and by inference, thymus-derived cells, are involved in the initiation of these particular responses (Section IB, IC and Coe, Feldman and Lee, 1966). Extrapolations from the results presented here may suggest that the sensitized effector cells in cell-mediated immune responses are bone marrow-derived and not of the same cell lineage as the cells which initiate the response (thymus derived). Short-lived mononuclear cells predominate in lesions of delayed hypersensitivity (discussed by Waksman, 1964) and, in passive transfer systems, the majority of these cells are derived from the bone marrow (Lubaroff and Waksman, 1968). These cells may or may not be specifically-sensitized effector cells. The majority of mononuclear cells most probably do not have specificity in the immunological sense and they apparently accumulate as a result of the liberation of pharmacologically-active substances such as the macrophage migration inhibitory factor (Bennett and Bloom, 1968). Obviously, the availability of techniques capable of enumerating sensitized effector cells in cell-mediated immunities must precede any type of reconstitution experiment, similar to those described in this thesis, and designed to test whether reconstitutive lymphoid cells provide effector cells or their precursors.

The complexity of the SRBC "antigen" has been stressed and a large inoculum of intravenously injected SRBC is unlikely to resemble the type and dose of antigenic material which the normal immune system is called upon to counteract. Furthermore, the humoral antibody response to SRBC is one of very few which is affected by neonatal thymectomy in the mouse. In view of these considerations, is there any reason to suppose that the primary immune response to many immunological cues involves the activity of more than one lymphoid cell type as seems to be the case in the primary 19S haemolysin response to SRBC in CBA mice?

Thymus-marrow interactions have been implicated in the response of mice to BSA (Taylor, 1968) and neonatal thymectomy depresses the response of mice to this antigen (Good et al., 1962; Taylor, 1963; Basch, 1966). Interestingly, neonatally thymectomy has little effect on the capacity of mice to respond to Salmonella flagellin (Martin and Miller, 1968b), and a thymus-marrow interaction cannot be demonstrated in irradiated mice (Armstrong and Diener, 1968). Likewise, the primary immune response to SRBC is unaffected by neonatal thymectomy in the rat (Pinnas and Fitch, 1966; Lind, P. and Steward, J., personal communications) and no evidence for a thymus-marrow interaction in irradiated rats has been obtained by McCullagh (1968). Many of the results are preliminary, however, and it would be unwise to emphasise this apparent separation of primary antibody responses into "thymus independent" (mediated by nonthymus-derived cells) and "thymus dependent" (mediated by cells, some of which are thymus-derived). Furthermore, the numerous differences in experimental design do not augur well for the validity of any comparison made between results obtained in various laboratories.

If recirculating thymus-derived lymphocytes are involved in some antibody responses but not others, it might be anticipated that adoptive transfers in irradiated recipients with normal thoracic duct lymphocytes may show differences depending on the antigen used.

However, the efficacy of thoracic duct cells in adoptively transferring antibody responses is not related in any simple fashion to the effects of neonatal thymectomy. Thus, whole thoracic duct lymphocyte inocula, collected on the first day of drainage, transfer primary responsiveness to (1) BSA in rats (Strober, 1968) - thymus dependent (Janković, Waksman and Arnason, 1962), (2) Salmonella flagellin in mice (Nossal, Shortman, Miller, Mitchell and Haskill, 1967) - thymus independent (Martin and Miller, 1968b), (3) SRBC in mice - thymus dependent (vide supra), (4) SRBC in rats (Section IC) - thymus independent, but not to (5) tetanus toxoid in rats (Strober, 1968) - thymus independent in mice (Hess et al., 1963; Hess and Stoner, 1966). A clearer picture will emerge with the development of techniques capable of separating or eliminating thymus-derived cells from peripheral lymphocyte populations.

One must predict on the basis of the partial immunological effects of neonatal thymectomy, that thymus-derived lymphocytes are unlikely to be involved in many primary antibody responses. Their participation must remain doubtful until it can be demonstrated that prenatal thymus cell migration occurs and that the number of thymus-derived cells is increased substantially by peripheral clonal expansion.

IV. BIBLIOGRAPHY

A. Personal

The majority of the experimental results in this thesis have been presented in the following publications:

Miller, J.F.A.P. and Mitchell, G. F. (1967a). Nature, 214, 994.

Miller, J.F.A.P. and Mitchell, G. F. (1967b). ibid, 216, 659.

Mitchell, G. F. and Miller, J.F.A.P. (1967). ibid, 214, 992.

Weiss, N. S., Mitchell, G. F. and Miller, J.F.A.P. (1967). ibid, 214, 995.

Miller, J.F.A.P. and Mitchell, G. F. (1968a). In "Advances in Transplantation", Dausset, J., Hamburger, J. and Mathé, G. (eds.). Munksgaard, Copenhagen. p. 79.

Miller, J.F.A.P., and Mitchell, G. F. (1968b). J. exp. Med., 128, 801.

Mitchell, G. F. and Miller, J.F.A.P. (1968). Proc. nat. Acad. Sci., 59, 296.

Mitchell, G. F. and Miller, J.F.A.P. (1968). J. exp. Med., 128, 821.

Nossal, G.J.V., Cunningham, A. J., Mitchell, G. F. and Miller, J.F.A.P. (1968). ibid, 128, 839.

Miller, J.F.A.P. and Mitchell, G. F. (1969a). In "Lymphatic Tissue and Germinal Centres in Immune Response", Advanc. exp. Med. Biol. Cottier, H. and Congdon, C. C. (eds.), Plenum Publishing Corp., New York. (in press).

Miller, J.F.A.P. and Mitchell, G. F. (1969b). Transplant. Rev., 1, (in press).

B. General

Adams, D. (1967). Acta haemat. (Basel), 37, 109.

Adner, M. M., Sherman, J. D. and Dameshek, W. (1965). Blood, 25, 511.

Agnew, H. D. (1967). Proc. Soc. exp. Biol. Med., 125, 132.

Aisenberg, A. C. (1967). J. exp. Med., 125, 833.

Aisenberg, A. C. and Davis, C. (1968). ibid, 128, 35.

Aisenberg, A. C. and Wilkes, B. (1965). Nature, 205, 716.

Aisenberg, A. C. and Wilkes, B. (1967). ibid, 213, 498.

Archer, O. K., Papermaster, B. W. and Good, R. A. (1964). In "The Thymus in Immunobiology. Structure, Function, and Role in Disease". Good, R. A. and Gabrielsen, A. E. (eds.). Hoeber-Harper, New York. p. 414.

Armstrong, W. D. and Diener, E. (1968). Annual Reports, Walter and Eliza Hall Inst. Med. Res., p. 43.

Arnason, B. G., de Vaux St Cyr, C. and Relyveld, E. H. (1964). Int. Arch. Allergy, 25, 206.

Arnason, B. G., de Vaux St Cyr, C. and Shaffner, J. B. (1964). J. Immunol., 93, 915.

Arnason, B. G., Janković, B. D. and Waksman, B. H. (1962). Blood, 20, 617.

Arnesen, K. (1958). Acta path. microbiol. scand., 43, 339.

Askonas, B. A. and Rhodes, J. M. (1965). Nature, 205, 470.

Askonas, B. A. and White, R. G. (1956). Brit. J. exp. Path., 37, 61.

Attardi, G., Cohn, M., Horibata, K. and Lennox, E. S. (1964). J. Immunol., 92, 346.

Auerbach, R. (1963). Science, 139, 1061.

August, C. S., Rosen, F. S., Filler, R. M., Janeway, C. A., Markowski, B. and Kay, H.E.M. (1968). Lancet, 2, 1210.

Austin, C. M. (1968). Aust. J. exp. Biol. med. Sci., 46, 581.

Azar, H. A. (1964). Proc. Soc. exp. Biol. Med., 116, 817.

Bailey, N.T.J. (1959). "Statistical Methods in Biology". The English Universities Press, London.

Bainbridge, D. R., Brent, L. and Gowland, G. (1966). Transplantation, 4, 138.

Baker, R. D. (1933). Anat. Rec., 55, 207.

Balfour, B. M., Cooper, E. H. and Meek, E. S. (1965). J. Reticuloendothel. Soc., 2, 379.

Ballantyne, B. and Burwell, R. G. (1965). Nature, 206, 1123.

Balner, H. and Dersjant, H. (1964). ibid, 204, 941.

Baney, R. N., Vazquez, J. J. and Dixon, F. J. (1962). Proc. Soc. exp. Biol. Med., 109, 1.

Barclay, T. J. (1964). Discussion. In "The Thymus". Wistar Inst. Symp. Monogr. No. 2. Defendi, V. and Metcalf, D. (eds.). The Wistar Inst. Press, Philadelphia. p. 117.

Barnes, D.W.H. and Loutit, J. F. (1967). Nature, 213, 1142.

Basch, R. S. (1966). Int. Arch. Allergy, 30, 105.

Benacerraf, B., Green, I. and Paul, W. E. (1967). Cold Spr. Harb. Symp. quant. Biol., 32, 569.

Bennett, B. and Bloom, B. R. (1968). Proc. nat. Acad. Sci., 59, 756.

Berembaum, M. C. (1966). Nature, 210, 41.

Bergland, K. (1965). In "Molecular and Cellular Basis of Antibody Formation". Šterzl, J. (ed.). Czech. Acad. Sci., Prague. p. 405.

Berglund, S. E., Markey, P. A. and Mergenhagen, S. E. (1967). Proc. Soc. exp. Biol. Med., 126, 84.

Berliner, D. L., Garzon, P., Lonngi, J. M. and Dougherty, T. F. (1967). J. Reticuloendothel. Soc., 4, 421.

Bernfield, M. R. and Fell, P. E. (1967). Proc. nat. Acad. Sci., 58, 2227.

Bierring, F. (1960). In "Haemopoiesis: Cell Production and its Regulation". Ciba Found. Symp. Wolstenholme, G.E.W. and O'Connor, M. (eds.). Churchill, London. p. 185.

Bierring, F. and Grunnet, I. (1964). Acta Anat. (Basel), 59, 182.

Biggart, J. D. (1966a). Brit. J. exp. Path., 47, 586.

Biggart, J. D. (1966b). ibid, 47, 590.

Billingham, R. E. (1963). Discussion. In "The Immunologically Competent Cell: Its Nature and Origin". Ciba Found. Study Gp. No. 16. Wolstenholme, G.E.W. and Knight, J. (eds.). Churchill, London, p. 29.

Billingham, R. E. (1967). In "The Harvey Lectures." Academic Press, New York and London. Ser. 62, p. 21.

Billingham, R. E. and Brent, L. (1956). Proc. roy. Soc. B., 146, 78.

Billingham, R. E. and Brent, L. (1959). Phil. Trans. roy. Soc. B., 242, 439.

Billingham, R. E., Brent, L. and Medawar, P. B. (1956). ibid, 239, 357.

Billingham, R. E., Defendi, V., Silvers, W. K. and Steinmuller, D. (1962). J. nat. Cancer Inst., 28, 365.

Billingham, R. E. and Medawar, P. B. (1951). J. exp. Biol., 28, 385.

Billingham, R. E. and Silvers, W. K. (1961). J. exp. Zool., 146, 113.

Billingham, R. E. and Silvers, W. K. (1964). In "The Thymus". Wistar Inst. Symp. Monogr. No. 2. Defendi, V. and Metcalf, D. (eds.). The Wistar Inst. Press, Philadelphia. p. 41.

Binns, R. M. and Hall, J. G. (1966). Brit. J. exp. Path., 47, 275.

Biozzi, G., Stiffel, C. and Mouton, D. (1966). In "Int. Symp. on Immunity, Cancer and Chemotherapy". Mihich, E. (ed.). Academic Press, New York. p, 103.

Bjørnboe, M., Gormsen, H. and Lundquist, A. R. (1947). J. Immunol., 55, 121.

Blau, J. N. and Waksman, B. H. (1964). Immunology, 7, 332.

Blau, J. N. and Gaugas, J. M. (1968). ibid, 14, 763.

Boak, J. L., Christie, G. H., Ford, W. L. and Howard, J. G. (1968). Proc. roy. Soc. B., 169, 307.

Boak, J. L. and Woodruff, M.F.A. (1965). Nature, 205, 396.

Bollman, J. L. (1948). J. Lab. clin. Med., 33, 1348.

Bollman, J. L., Cain, J. C. and Gridlay, J. H. (1948). ibid, 33, 1349.

Bos, W. H. (1967). "Recirculatie en transformatie van lymphocyten." M.D. Thesis, Groningen.

Boyse, E. A., Miyazawa, M., Aoki, T. and Old, L. J. (1968). Proc. roy. Soc. B., 170, 175.

Braun, W. and Nakano, M. (1965). Proc. Soc. exp. Biol. Med., 119, 701.

Braunsteiner, H., Höfer, R. and Sailer, S. (1961). Germ. Med. Monthly, 6, 273.

Brent, L. and Medawar, P. (1966). Proc. roy. Soc. B., 165, 281.

Bretscher, P. A. and Cohn, M. (1968). Nature, 220, 444.

Bryant, B. J. (1963). J. Cell Biol., 18, 515.

Brumby, M. and Metcalf, D. (1967). Proc. Soc. exp. Biol. Med., 124, 99.

Buckton, K. E., Jacobs, P. A., Court Brown, W. M. and Doll, R. (1962). Lancet, 2, 676.

Buckton, K. E. and Pike, M. C. (1964). Int. J. Radiat. Biol., 8, 439.

Burch, P.R.J. and Burwell, R. G. (1965). Quart. Rev. Biol., 40, 252.

Burnet, F. M. (1959). "The Clonal Selection Theory of Acquired Immunity". Cambridge University Press, London.

Burnet, F. M. (1968a). Nature, 218, 426.

Burnet, F. M. (1968b). Lancet, 2, 610.

Burnet, F. M. and Holmes, M. C. (1962). Nature, 194, 146.

Burnet, F. M. and Holmes, M. C. (1964). J. Path. Bact., 88, 229.

Burwell, R. G. (1962). Ann. N.Y. Acad. Sci., 99, 821.

Bussard, A. E. (1966). Science, 153, 887.

Bussard, A. E. and Lurie, M. (1967). J. exp. Med., 125, 873.

Caffrey, R. W., Rieke, R. O. and Everett, N. B. (1962). Acta haemat. (Basel), 28, 145.

Cain, W. A., Cooper, M. D. and Good, R. A. (1968). Nature, 217, 87.

Celada, F. (1967). J. exp. Med., 125, 199.

Celada, F. and Carter, R. R. (1962). J. Immunol., 89, 161.

Celada, F. and Klein, G. (1967). Nature, 215, 1136.

Celada, F. and Wigzell, H. (1966). Immunology, 10, 231.

Chaperon, E. A. and Claman, H. N. (1967). Fed. Proc., 26, 640.

Chaperon, E. A., Selner, J. C. and Claman, H. N. (1968). Immunology, 14, 553.

Chapman, N. D. and Dutton, R. W. (1965). J. exp. Med., 121, 85.

Cheng, V. and Trentin, J. J. (1967a). Proc. Soc. exp. Biol. Med., 126, 467.

Cheng, V. and Trentin, J. J. (1967b). Fed. Proc., 26, 641.

Cherry, C. P., Eisenstein, R. and Gluckmann, A. (1967). Brit. J. exp. Path., 48, 90.

Chou, C-T., Dubiski, S., and Cinader, B. (1967). J. exp. Med., 126, 305.

Christensen, B. C. and Ottesen, J. (1955). Acta haemat. (Basel), 13, 289.

Claflin, A. J. and Smithies, O. (1967). Science, 157, 1561.

Claman, H. N. (1966). Proc. Soc. exp. Biol. Med., 121, 236.

Claman, H. N. and Brunstetter, F. H. (1968). J. Immunol., 100, 1127.

Claman, H. N., Chaperon, E. A. and Selner, J. C. (1968). Proc. Soc. exp. Biol. Med., 127, 462.

Claman, H. N., Chaperon, E. A. and Triplett, R. F. (1966a). Proc. Soc. exp. Biol. Med., 122, 1167.

Claman, H. N., Chaperon, E. A. and Triplett, R. F. (1966b). J. Immunol., 97, 828.

Clark, S. L. Jr. (1966). In "Thymus. Experimental and Clinical Studies". Ciba Found. Symp. Wolstenholme, G.E.W. and Porter, R. (eds.). Churchill, London. p. 3.

Clark, S. L. Jr. (1968). J. exp. Med., 128, 927.

Clark, W.G.B., Williams, C. B. and Yoffey, J. M. (1966). J. Lab. clin. Med., 67, 439.

Coassin, N. A. and Kline, D. L. (1957). Amer. J. Physiol., 190, 147.

Coe, J. E., Feldman, J. D. and Lee, S. (1966). J. exp. Med., 123, 267.

Cohen, E. P. and Talmage, D. W. (1965). ibid, 121, 125.

Cohen, M. W., Thorbecke, G. J., Hochwald, G. and Jacobson, E. B. (1963). Proc. Soc. exp. Biol. Med., 114, 242.

Congdon, C. C. and Duda, D. B. (1961). Arch. Path., 71, 311.

Cooper, M. D., Peterson, R.D.A., South, M. A. and Good, R. A. (1966). J. exp. Med., 123, 75.

Corsi, A. and Giusti, G. V. (1967a). Nature, 216, 493.

Corsi, A. and Giusti, G. V. (1967b). ibid, 213, 618.

Cottier, H., Cronkite, E. P., Jansen, C. R., Rai, K. R., Singer, S. and Sipe, C. R. (1964). Blood, 24, 241.

Court Brown, W. M., Buckton, K. E. and McLean, A. S. (1965). Lancet, 1, 1239.

Craddock, C. G. (1965). Acta haemat. (Basel), 33, 19.

Craddock, C. G., Nakai, G. S., Fukuta, H. and Vanslager, L. M. (1964). J. exp. Med., 120, 389.

Craddock, C. G., Rytömaa, T. and Nakai, G. S. (1964). In "The Thymus in Immunobiology. Structure, Function, and Role in Disease". Good, R. A. and Gabrielsen, A. E. (eds.). Hoeber-Harper, New York. p. 317.

Craddock, C. G., Winkelstein, A., Matsuyuki, Y. and Lawrence, J. S. (1967a). In "Control of Cellular Growth in Adult Organisms". Teir, H. and Rytömaa, T. (eds.). Academic Press, London. p. 153.

Craddock, C. G., Winkelstein, A., Matsuyuki, Y. and Lawrence, J. S. (1967b). J. exp. Med., 125, 1149.

Cronkite, E. P., Bond, V. P., Fliedner, T. M. and Rubini, J. R. (1959). Lab. Invest., 8, 263.

Cronkite, E. P., Jansen, C. R., Cottier, H., Rai, K. and Sipe, C. R. (1964). Ann. N.Y. Acad. Sci., 113, 566.

Cross, A. M., Davies, A.J.S., Doe, B. and Leuchars, E. (1964). J. exp. Med., 119, 837.

Cudkowicz, G. (1965). In "Isoantigens and Cell Interactions". Palm, J. (ed.). Wistar Inst. Symp. Monogr. No. 3., Wistar Inst. Press. Philadelphia. p. 37.

Cunningham, A. J. (1968a). Aust. J. exp. Biol. Med. Sci., 46, 141.

Cunningham, A. J. (1968b). Annual Reports, Walter and Eliza Hall Inst. of Med. Res., p. 39.

Cunningham, A. J. and Szenberg, A. (1968). Immunology, 14, 599.

Dalmasso, A. P., Martinez, C., Sjodin, K. and Good, R. A. (1963). J. exp. Med., 118, 1089.

Davies, A.J.S., Festenstein, H., Leuchars, E., Wallis, V. J. and Doenhoeff, M. J. (1968a). Lancet, 1, 183.

Davies, A.J.S., Leuchars, E., Wallis, V. and Koller, P. C. (1966). Transplantation, 4, 438.

Davies, A.J.S., Leuchars, E., Wallis, V., Marchant, R. and Elliott, E. V. (1967). ibid, 5, 222.

Davies, A.J.S., Leuchars, E., Wallis, V., Sinclair, N. R. St C. and Elliott, E. V. (1968b). In "Advances in Transplantation". Dausset, J., Hamburger, J. and Mathé, G. (eds.). Munksgaard, Copenhagen, p. 97.

Defendi, V., and Manson, L. A. (1963). Nature, 198, 359.

Dent, P. B. and Good, R. A. (1965). ibid, 207, 491.

Dent, P. B. and Peterson, R.D.A. (1967). Fed. Proc., 26, 621.

Dietrich, F. M. and Dukor, P. (1967). Pathol. Microbiol. (Basel), 30, 909.

Dixon, F. J., Weigle, W. O. and Roberts, J. C. (1957). J. Immunol., 78, 56.

Doria, G., Goodman, J. W., Gengozian, N. and Congdon, C. C. (1962). ibid, 88, 20.

Dougherty, T. F. (1952). Physiol. Rev., 32, 379.

Dubert, A. and Kaplan, H. S. (1961). Fed. Proc., 20, 40.

Duhig, J. T. (1965). Nature, 207, 651.

Dukor, P. and Dietrich, F. M. (1967). Int. Arch. Allergy, 32, 521.

Dukor, P., Dietrich, F. M. and Rosenthal, M. (1966). Clin. exp. Immunol., 1, 391.

Dukor, P., Miller, J.F.A.P., House, W. and Allman, V. (1965). Transplantation, 3, 639.

Dukor, P., Miller, J.F.A.P. and Sacquet, E. (1968). Clin. exp. Immunol., 3, 191.

Duplan, J. F. (1965). Ann. Inst. Pasteur, 108, 1.

Duplan, J. F., Foschi, G. V. and Manson, L. A. (1962). Proc. Soc. exp. Biol. Med., 110, 426.

Dutton, R. W. and Harris, G. (1963). Fed. Proc., 22, 266.

Dutton, R. W. and Mishell, R. I. (1967a). J. exp. Med., 126, 443.

Dutton, R. W. and Mishell, R. I. (1967b). Cold Spr. Harb. Symp. quant. Biol., 32, 407.

East, J. and Parrott, D.M.V. (1964). J. nat. Cancer Inst., 33, 673.

East, J., Parrott, D.M.V., Chesterman, F. C. and Pomerance, A. (1963). J. exp. Med., 118, 1069.

Ehrich, W. E. and Harris, T. N. (1942). ibid, 76, 335.

Eidinger, D. and Pross, H. F. (1967). ibid, 126, 15 .

Eisen, H. N. and Karush, F. (1964). Nature, 202, 677.

Elders, M. J., Parkham, B. A. and Hughes, E. R. (1968). J. exp. Med., 127, 649.

Ellis, S. T., Gowans, J. L. and Howard, J. C. (1967). Cold Spr. Harb. Symp. quant. Biol., 32, 395.

Elves, M. W. (1966). "The Lymphocytes". Lloyd-Luke, London.

Elves, M. W., Roath, S. and Israels, M.C.G. (1964). Brit. med. J., 2, 1051.

Ernström, U., Gyllensten, L. and Larssön, B. (1965). Nature, 207, 540.

Ernström, U. and Larssön, B. (1966). Acta path. microbiol. scand., 67, 267.

Everett, N. B. (1964). Discussion. In "The Thymus in Immunobiology. Structure, Function and Role in Disease". Good, R. A. and Gabrielsen, A. E. (eds.). Hoeber-Harper, New York. p. 287.

Everett, N. B., Caffrey, R. W. and Rieke, W. O. (1964). Ann. N.Y. Acad. Sci., 113, 887.

Everett, N. B., Rienhardt, W. O., and Yoffey, J. M. (1960). Blood, 15, 82.

Everett, N. B. and Tyler, R. W. (1967). Int. Rev. Cytol., 22, 205.

Fagraeus, A. (1948). Acta med. scand., 130, (suppl. 204), 3.

Fahey, J. L., Barth, W. F. and Law, L. W. (1965). J. nat. Cancer Inst., 35, 663.

Feldman, M. and Gallily, R. (1967). Cold Spr. Harb. Symp. quant. Biol., 32, 415.

Feldman, M. and Globerson, A. (1964). Ann. N.Y. Acad. Sci., 120, 182.

Fichtelius, K. E. (1968). Exp. hemat., No. 17, (iii).

Fichtelius, K. E. (1960). In "Haemopoiesis. Cell Production and its Regulation." Ciba Found. Symp. Wolstenholme, G.E.W. and O'Connor, M. (eds.). Churchill, London. p. 204.

Fichtelius, K. E. and Bryant, B. J. (1964). In "The Thymus in Immunobiology. Structure, Function, and Role in Disease". Good, R. A. and Gabrielsen, A. E. (eds.). Hoeber-Harper, New York. p. 274.

Fichtelius, K. E. and Linna, J. (1967). In "Control of Cellular Growth in Adult Organisms". Tier, H. and Rytömaa, T. (eds.). Academic Press, London. p. 171.

Fiore-Donati, L., Chieco-Bianchi, L., de Benedictis, G. and Tridente, G. (1964). Blood, 24, 770.

Fishman, M. and Adler, F. L. (1963). J. exp. Med., 117, 595.

Fishman, M. and Adler, F. L. (1967). Cold Spr. Harb. Symp. quant. Biol., 32, 343.

Fitch, F. W. and Rowley, D. A. (1967). Fed. Proc., 26, 641.

Fitch, F. W., Stejskal, R. and Rowley, D. A. (1969). In "Lymphatic Tissue and Germinal Centers in Immune Response". Advanc. exp. Med. Biol. Cottier, H. and Congdon, C. C. (eds.). Plenum Publishing Corp., New York (in press).

Fitzgerald, P. H. (1964). J. Theoret. Biol., 6, 13.

Fliedner, T. M., Kesse, M., Cronkite, E. P. and Robertson, J. S. (1964). Ann. N.Y. Acad. Sci., 113, 578.

Ford, C. E. (1966a). In "Thymus. Experimental and Clinical Studies". Wolstenholme, G.E.W. and Porter, R. (eds.). Churchill, London. p. 131.

Ford, C. E. (1966b). Appendix I to "Tissue Grafting and Radiation. Micklem, H. S. and Loutit, J. F. (eds.). Academic Press, New York. p. 197.

Ford, C. E. and Micklem, H. S. (1963). Lancet, 1, 359.

Ford, W. L. (1967). Brit. J. exp. Path., 48, 335.

Ford, W. L. (1968). Immunology, 15, 609.

Ford, W. L. and Gowans, J. L. (1967). Proc. roy. Soc. B., 168, 244.

Ford, W. L., Gowans, J. L. and McCullagh, P. J. (1966). In "Thymus. Experimental and Clinical Studies". Ciba Found. Symp. Wolstenholme, G.E.W. and Porter, R. (eds.). Churchill, London. p. 58.

Friedman, H. (1964). Proc. Soc. exp. Biol. Med., 117, 526.

Friedman, H. P., Stavitsky, A. B. and Solomon, J. M. (1965). Science, 149, 1106.

Fudenberg, H. H. and Hirschorn, K. (1964). ibid, 145, 611.

Furth, R. van and Cohn, Z. A. (1968). J. exp. Med., 128, 415.

Furth, R. van, Schuit, H.R.E. and Hijmans, W. (1966a). Immunology, 11, 19.

Furth, R. van, Schuit, H.R.E. and Hijmans, W. (1966b). ibid, 11, 29.

Gallily, R. and Feldman, M. (1967). Immunology, 12, 197.

Galton, M. and Reed, P. B. (1966). Transplantation, 4, 168.

Gengozian, N., Makinodan, T. and Shekarchi, I. C. (1961). J. Immunol., 86, 113.

Gershon, R. K., Wallis, V., Davies, A.J.S. and Leuchars, E. (1968). Nature, 218, 380.

Gesner, B. M. and Ginsburg, V. (1964). Proc. nat. Acad. Sci., 52, 750.

Gesner, B. M. and Gowans, J. L. (1962a). Brit. J. exp. Path., 43, 424.

Gesner, B. M. and Gowans, J. L. (1962b). ibid, 43, 431.

Globerson, A. (1966). J. exp. Med., 123, 25.

Globerson, A. and Auerbach, R. (1967). ibid, 126, 223.

Globerson, A. and Feldman, M. (1964). Transplantation, 2, 212.

Goedbloed, J. F. and Vos, O. (1965). ibid, 3, 368.

Goldschneider, I. and McGregor, D. D. (1966). Nature, 212, 1433.

Goldschneider, I. and McGregor, D. D. (1968a). J. exp. Med., 127, 155.

Goldschneider, I. and McGregor, D. D. (1968b). Lab. Invest., 18, 397.

Goldstein, G. and MacKay, I. R. (1969). "The Human Thymus". William Heinemann, London. (in press).

Goldstein, A. L., Slater, F. D. and White, A. (1966). Proc. nat. Acad. Sci., 56, 1010.

Good, R. A. (1966). Discussion. In "Thymus. Experimental and Clinical Studies". Ciba Found. Symp. Wolstenholme, G.E.W., and Porter, R. (eds.). Churchill, London. p. 468.

Good, R. A., Dalmasso, A. P., Martinez, C., Archer, O. K., Pierce, J. C. and Papermaster, B. W. (1962). J. exp. Med., 116, 773.

Goodman, J. W. and Hodgson, G. S. (1962). Blood, 19, 702.

Gordon, H. A. (1959). Ann. N.Y. Acad. Sci., 78, 208.

Gottlieb, A. A., Glisin, V. R. and Doty, P. (1967). Proc. nat. Acad. Sci., 57, 1849.

Gowans, J. L. (1957). Brit. J. exp. Path., 38, 67.

Gowans, J. L. (1959a). J. Physiol., 146, 54.

Gowans, J. L. (1959b). In "Kinetics of Cellular Proliferation". Stohlman, F. Jr. (ed.). Grune and Stratton, New York, p. 64.

Gowans, J. L. (1962). Ann. N.Y. Acad. Sci., 99, 432.

Gowans, J. L. (1964). Discussion. In "The Thymus in Immunobiology. Structure, Function, and Role in Disease." Good, R. A. and Gabrielsen, A. E. (eds.). Hoeber-Harper, New York. p. 287.

Gowans, J. L. (1966). Int. Rev. exp. Path., 5, 1.

Gowans, J. L. and Knight, E. J. (1964). Proc. roy. Soc. B., 159, 257.

Gowans, J. L. and McGregor, D. D. (1965). Progr. Allergy, 9, 1.

Gowans, J. L., McGregor, D. D. and Cowen, D. M. (1963). In "The Immunologically Competent Cell: Its Nature and Origin". Ciba Found. Study Gp. No. 16. Wolstenholme, G.E.W. and Knight, J. (eds.). Churchill, London. p. 20.

Gowans, J. L., McGregor, D. D., Cowen, D. M. and Ford, C. E. (1962). Nature, 196, 651.

Gowans, J. L. and Uhr, J. W. (1966). J. exp. Med., 124, 1017.

Greaves, M. F., Roitt, I. M. and Rose, M. E. (1968). Nature, 220, 293.

Green, I. (1964). J. exp. Med., 119, 581.

Green, I., Vassalli, P., Nussenzweig, V. and Benacerraf, B. (1967). ibid, 125, 511.

Grégoire, Ch. (1958). Quart. J. micr. Sci., 99, 511.

Grégoire, Ch. and Duchâteau, G. (1956). Arch. Biol. (Liege), 67/2, 269.

Gregory, C. J. and Lajtha, L. (1968). Nature, 218, 1079.

Hall, J. G. and Morris, B. (1964). Lancet, 1, 1077.

Hall, J. G. and Morris, B. (1965a). J. exp. Med., 121, 901.

Hall, J. G. and Morris, B. (1965b). Brit. J. exp. Path., 46, 450.

Hamilton, L. D. (1954). J. clin. Invest., 33, 939.

Hamilton, L. D. (1956). Nature, 178, 597.

Hand, T., Castor, P. and Luckey, T. D. (1967). Biochem. biophys. Res. Commun., 26, 18.

Harris, J. E. and Ford, C. E. (1964). Nature, 201, 884.

Harris, J. E., Ford, C. E., Barnes, D.W.H. and Evans, E. P. (1964). ibid, 201, 886.

Harris, S. and Harris, T. N. (1955). J. Immunol., 74, 318.

Harris, S., Harris, T. N., and Ogburn, C. A. (1967). ibid, 99, 447.

Harris, T. N. and Harris, S. (1960). In "Cellular Aspects of Immunity". Ciba Found. Symp. Wolstenholme, G.E.W. and O'Connor, M. (eds.). Churchill, London. p. 172.

Harris, T. N., Hummeler, K. and Harris, S. (1966). J. exp. Med., 123, 161.

Harris, T. N., Rhoads, J. and Stokes, J. Jr. (1948). J. Immunol., 58, 27.

Harven, E. de (1964). J. exp. Med., 120, 857.

Hays, E. F. (1964). Proc. Soc. exp. Biol. Med., 117, 45.

Hays, E. F. (1967). Blood, 29, 29.

Heath, T. (1964). Nature, 203, 1296.

Hechtel, M., Dishon, T. and Braun, W. (1965). Proc. Soc. exp. Biol. Med., 120, 728.

Hege, J. S. and Cole, L. J. (1966). J. Immunol., 96, 559.

Hege, J. S. and Cole, L. J. (1967). ibid, 99, 61.

Hellström, K. E. and Möller, G. (1965). Progr. Allergy, 9, 158.

Hemphill, F. E., Segre, D. and Myers, W. L. (1966). Proc. Soc. exp. Biol. Med., 123, 265.

Henry, C. and Jerne, N. K. (1968). J. exp. Med., 128, 133.

Hess, M. W., Cottier, H. and Stoner, R. D. (1963). J. Immunol., 91, 425.

Hess, M. W. and Stoner, R. D. (1966). Int. Arch. Allergy, 30, 37.

Hilgard, H. R., Yunis, E. J., Sjodin, K., Martinez, C. and Good, R. A. (1964). Nature, 202, 668.

Hiramoto, R. N., Hamlin, N. M. and Harris, H. L. (1968). J. Immunol., 100, 637.

Hirata-Hibi, M. (1967). J. Reticuloendothel. Soc., 4, 370.

Holub, M., Říha, I. and Kamarýtová, V. (1965). In "Molecular and Cellular Basis of Antibody Formation". Šterzl, J. (ed.). Czech. Acad. Sci., Prague, p. 447.

Horiuchi, A. and Waksman, B. H. (1968). J. Immunol., 100, 974.

Horvàth, G. (1963). Nature, 200, 185.

Houba, V. and Houba, J. (1966). Experentia (Basel), 22, 829.

Hudson, G. and Yoffey, J. M. (1966). Proc. roy. Soc. B., 165, 486.

Hulse, E. V. (1963). Brit. J. Haemat., 9, 265.

Humble, J. G., Jayne, W.H.W. and Pulvertaft, R.J.V. (1956). ibid, 2, 283.

Hummeler, K., Harris, T. N., Tomassini, N., Hechtel, M. and Farber, M. B. (1966). J. exp. Med., 124, 255.

Humphrey, J. H. (1967). Brit. med. Bull., 23, 93.

Humphrey, J. H., Parrott, D.M.V. and East, J. (1964). Immunology, 7, 419.

Hungerford, D. A., Donnelly, A. J., Nowell, P. C. and Beck, S. (1959). Amer. J. hum. Genet., 11, 215.

Huvos, A. G., Cali, A. and Azar, H. A. (1966). Amer. J. Path., 48, 627.

Ingraham, J. S. and Bussard, A. (1964). J. exp. Med., 119, 667.

Isaković, K., Waksman, B. H. and Wennersten, C. (1965). J. Immunol., 95, 602.

Jacob, F. (1960). In "The Harvey Lectures". Academic Press, New York. p. 1.

Janković, B. D. and Leskowitz, S. (1965). Proc. Soc. exp. Biol. Med., 118, 1164.

Janković, B. D., Waksman, B. H. and Arnason, B. G. (1962). J. exp. Med., 116, 159.

Jaroslow, B. N. (1960). J. infect. Dis., 107, 56.

Jaroslow, B. N. and Taliaferro, W. H. (1956). ibid, 98, 75.

Jenkin, C. R. and Rowley, D. (1961). J. exp. Med., 114, 363.

Jerne, N. K. (1967a). Discussion. Cold Spr. Harb. Symp. quant. Biol., 32, 406.

Jerne, N. K. (1967b). Discussion. ibid, 32, 460.

Jerne, N. K. (1967c). Waiting for the end. ibid, 32, 591.

Jerne, N. K. and Nordin, A. A. (1963). Science, 140, 405.

Jerne, N. K., Nordin, A. A. and Henry, C. (1963). In "Cell-Bound Antibodies". Amos, B. and Koprowski, H. (eds.). Wistar Inst. Press, Philadelphia. p. 109.

Kaplan, H. S. (1966). Discussion. In "Thymus. Experimental and Clinical Studies". Ciba Found. Symp. Wolstenholme, G.E.W. and Porter, R. (eds.). Churchill, London. p. 176.

Kaplan, H. S. and Brown, M. B. (1957). In "The Leukaemias: Etiology, Pathophysiology and Treatment". Rebuck, J. W., Bethell, F. H. and Monte, R. W. (eds.). Academic Press, New York. p. 163.

Kaplan, H. S. and Rosston, B. H. (1959). Transplant. Bull., 6, 107.

Kawashima, Y., Sugimura, M., Hwang, Y-C. and Kudo, N. (1964). Jap. J. vet. Res., 12, 69.

Kearney, R. and Halliday, W. J. (1965). J. Immunol., 95, 109.

Kennedy, J. C., Siminovitch, L., Till, J. E. and McCulloch, E. A. (1965a). Proc. Soc. exp. Biol. Med., 120, 868.

Kennedy, J. C., Till, J. E., Siminovitch, L. and McCulloch, (1965b). J. Immunol., 94, 715.

Kennedy, J. C., Till, J. E., Siminovitch, L. and McCulloch, E. A. (1966). ibid, 96, 973.

Keuning, F. J., Meer, J. van der, Nieuwenhuis, P. and Oudendijk, P. (1963). Lab. Invest., 12, 156.

Kind, P. and Campbell, P. A. (1968). J. Immunol., 100, 55.

Klein, J. J., Goldstein, A. L. and White, A. (1965). Proc. nat. Acad. Sci, 53, 812.

Knight, S., Bradley, J., Oppenheim, J. J. and Ling, N. R. (1968). Clin. exp. Immunol., 3, 323.

Koros, A. M., Mazur, J. M. and Mowery, M. J. (1968). J. exp. Med., 128, 235.

Kotani, M., Seiki, K., Yamashita, A. and Horii, I. (1966). Blood, 27, 511.

Krüger, G. (1968). J. nat. Cancer Inst., 41, 287.

Lajtha, L. (1960). Discussion. In "Haemopoiesis. Cell Production and its Regulation". Wolstenholme, G.E.W. and O'Connor, M. (eds.). Ciba Found. Symp. Churchill, London. p. 220.

Landsteiner, K. and Chase, M. W. (1942). Proc. Soc. exp. Biol. Med., 49, 688.

Landy, M., Sanderson, R. P., Bernstein, M. T. and Lerner, E. M. II. (1965). Science, 147, 1591.

Laskov, R. (1968). Nature, 219, 973.

Law, L. W. (1966a). Cancer Res., 26, 551.

Law, L. W. (1966b). Nature, 210, 1118.

Law, L. W. and Agnew, H. D. (1968). Proc. Soc. exp. Biol. Med., 127, 953.

Law, L. W., Dunn, T. B., Trainin, N. and Levey, R. H. (1964). In "The Thymus". Wistar Inst. Monogr. No. 2. Defendi, V. and Metcalf, D. (eds.). Wistar Inst. Press, Philadelphia. p. 105.

Law, L. W., Trainin, N., Levey, R. H. and Barth, W. F. (1964). Science, 143, 1049.

Lawrence, H. S. (1967). Introductory remarks. Symposium on in vitro correlates of delayed hypersensitivity. Fed. Proc., 27, 3.

Leduc, E. H., Coons, A. H. and Connolly, J. M. (1955). J. exp. Med., 102, 61.

Leuchars, E., Cross, A. M., Davies, A.J.S. and Wallis, V. J. (1964). Nature, 203, 1189.

Leuchars, E., Cross, A. M. and Dukor, P. (1965). Transplantation, 3, 28.

Leuchars, E., Morgan, A., Davies, A.J.S. and Wallis, V. J. (1967). Nature, 214, 801.

Levey, R. H., Trainin, N. and Law, L. W. (1963). J. nat. Cancer Inst., 31, 199.

Levey, R. H., Trainin, N., Law, L. W., Black, P. H. and Rowe, W. P. (1963). Science, 142, 483.

Levine, B. B. (1965). J. exp. Med., 121, 873.

Linna, T. J. (1967). Acta path. microbiol. scand., 71, 68.

Linna, T. J. (1968). Blood, 32, 727.

Lischner, H. W., Punnett, H. H. and DiGeorge, A. M. (1967). Nature, 214, 580.

Litt, M. (1967). Cold Spr. Harb. Symp. quant. Biol., 32, 477.

Little, J. R., Brecher, G., Bradley, T. R. and Rose, S. (1962). Blood, 19, 236.

Loutit, J. F. (1962). Lancet, 2, 1106.

Lubaroff, D. M. and Waksman, B. H. (1968). J. exp. Med., 128, 1437.

Makinodan, T. and Albright, J. F. (1963). In "Immunopathology". Grabar, P. and Miescher, P. A. (eds.). Schwabe and Co., Basel. p. 99.

Makinodan, T. and Albright, J. F. (1967). Progr. Allergy, 10, 1.

Makinodan, T., Gengozian, N. and Shekarchi, I. C. (1958). J. nat. Cancer Inst., 20, 591.

Makinodan, T., Kastenbaum, M. A. and Peterson, W. J. (1962). J. Immunol., 88, 31.

Malaviya, A. and Tannenberg, W.J.K. (1967). Fed. Proc., 26, 751.

Mandel, M. A. (1967). Proc. Soc. exp. Biol. Med., 126, 521.

Mann, J. D. and Higgins, G. M. (1950). Blood, 5, 177.

Marchesi, V. T. and Gowans, J. L. (1964). Proc. roy. Soc. B., 159, 283.

Marshall, A.H.E. and White, R. G. (1961). Brit. J. exp. Path., 42, 379.

Martin, W. J. and Miller, J.F.A.P. (1968a). J. exp. Med., 128, 855.

Martin, W. J. and Miller, J.F.A.P. (1968b). Annual Reports. Walter and Eliza Hall Inst. Med. Res. p. 77.

Matsuyama, M., Wiadrowski, M. N. and Metcalf, D. (1966). J. exp. Med., 123, 559.

Medawar, P. B. (1963). In "The Immunologically Competent Cell: Its Nature and Origin". Ciba Found. Study Gp. No. 16. Wolstenholme, G.E.W. and Knight, J. (eds.). Churchill, London. p. 1.

Metcalf, D. (1956). Brit. J. Cancer, 10, 442.

Metcalf, D. (1960). Brit. J. Haemat., 6, 324.

Metcalf, D. (1963). Aust. J. exp. Biol. med. Sci., 41, 437.

Metcalf, D. (1964). In "The Thymus". Wistar Inst. Symp. Monogr. No. 2. Defendi, V. and Metcalf, D. (eds.). Wistar Inst. Press, Philadelphia. p. 53.

Metcalf, D. (1965). Nature, 208, 1336.

Metcalf, D. (1966). In "Thymus, Experimental and Clinical Studies". Ciba Found. Symp. Wolstenholme, G.E.W. and Porter, R. (eds.). Churchill, London. p. 242.

Metcalf, D. (1967). Cold Spr. Harb. Symp. quant. Biol., 32, 583.

Metcalf, D. (1968). J. cell. Physiol., 72, 9.

Metcalf, D. and Brumby, M. (1966). J. cell. Physiol., 67 (Suppl. 1), 149.

Metcalf, D., Sparrow, N., Nakamura, K. and Ishidate, M. (1961). Aust. J. exp. Biol. med. Sci., 39, 441.

Metcalf, D. and Wakonig-Vaartaja, R. (1964). Proc. Soc. exp. Biol. Med., 115, 731.

Metcalf, W. K. and Osmond, D. G. (1966). Exp. Cell Res., 41, 669.

Meuwissen, H., Bach, F., VanAlten, P. and Good, R. A. (1967). Fed. Proc., 26, 477.

Micklem, H. S., Ford, C. E., Evans, E. P. and Gray, J. (1966). Proc. roy. Soc. B., 165, 78.

Miller, J.F.A.P. (1960). Brit. J. Cancer, 14, 93.

Miller, J.F.A.P. (1961). Lancet, 2, 748.

Miller, J.F.A.P. (1962a). Proc. roy. Soc. B., 156, 415.

Miller, J.F.A.P. (1962b). Ann. N.Y. Acad. Sci., 99, 340.

Miller, J.F.A.P. (1963). Brit. med. J., 2, 459.

Miller, J.F.A.P. (1964a). In "The Thymus in Immunobiology. Structure, Function, and Role in Disease". Good, R. A. and Gabrielsen, A. E. (eds.). Hoeber-Harper, New York. p. 436.

Miller, J.F.A.P. (1964b). Science, 144, 1544.

Miller, J.F.A.P. (1965). Nature, 208, 1337.

Miller, J.F.A.P. (1968). In "The Biological Basis of Medicine". Bittar, E. E. (ed.). Academic Press, London. p. 265.

Miller, J.F.A.P., Block, M., Rowlands, D. T. Jr. and Kind, P. (1965). Proc. Soc. exp. Biol. Med., 118, 916.

Miller, J.F.A.P., Burgh, P. M. de, Dukor, P., Grant, G., Allman, V. and House, W. (1966). Clin. exp. Immunol., 1, 61.

Miller, J.F.A.P., Burgh, P. M. de and Grant, G. A. (1965). Nature, 208, 1332.

Miller, J.F.A.P. and Davies, A.J.S. (1964). Ann. Rev. Med., 15, 23.

Miller, J.F.A.P., Dukor, P., Grant, G. A., Sinclair, N. R. St C. and Sacquet, E. (1967). Clin. exp. Immunol., 2, 513.

Miller, J.F.A.P., Howard, J. G. (1964). J. Reticuloendothel. Soc., 1, 369.

Miller, J.F.A.P., Leuchars, E., Cross, A. M. and Dukor, P. (1964). Ann. N.Y. Acad. Sci., 120, 205.

Miller, J.F.A.P. and Osoba, D. (1967). Physiol. Rev., 47, 437.

Miller, J.F.A.P., Osoba, D. and Dukor, P. (1965). Ann. N.Y. Acad. Sci., 124, 95.

Miller, J. J. III (1964). J. Immunol., 92, 673.

Mims, C. A. (1962). Brit. J. exp. Path., 43, 639.

Mishell, R. I. and Dutton, R. W. (1967). J. exp. Med., 126, 423.

Mitchell, J., McDonald, W. and Nossal, G.J.V. (1963). Aust. J. exp. Biol. med. Sci., 41, 411.

Mitchison, N. A. (1957). J. cell. comp. Physiol., 50 (Suppl. 1), 247.

Mitchison, N. A. (1967). Cold Spr. Harb. Symp. quant. Biol., 32, 431.

Möller, G. and Möller, E. (1966). Ann. N.Y. Acad. Sci., 129, 735.

Moore, M.A.S. and Owen, J.J.T. (1967). Lancet, 2, 658.

Mori, R., Takeya, K., Minamishima, Y. and Tasaki, T. (1965). Proc. Jap. Acad., 41, 975.

Morris, B. (1968). Oral Presentation. Meeting of Australian Society of Immunologists, Canberra.

Morse, S. I. and Riester, S. K. (1967). J. exp. Med., 125, 619.

Mosier, D. E. and Cohen, E. P. (1968). Nature, 219, 969.

Mosier, D. E. and Coppleson, L. W. (1968). Proc. nat. Acad. Sci., 61, 542.

Murray, R. G. and Murray, A. (1961). Blood, 18, 737.

Murray, R. G. and Woods, P. A. (1964). Anat. Rec., 150, 113.

McBride, R. A. (1966). Cancer Res., 26, 1135.

McCullagh, P. J. (1968). Meeting of Australian Society of Immunologists. Canberra. Oral Presentation.

McCulloch, E. A. and Till, J. E. (1963). J. cell. comp. Physiol., 61, 301.

McGregor, D. D. (1966). Fed. Proc., 25, 1713.

McGregor, D. D. (1968). J. exp. Med., 127, 953.

McGregor, D. D., McCullagh, P. J. and Gowans, J. L. (1967). Proc. roy. Soc. B., 168, 229.

McGregor, D. D. and Gowans, J. L. (1963). J. exp. Med., 117, 303.

McGregor, D. D. and Gowans, J. L. (1964). Lancet, 1, 629.

McIntire, K. R., Sell, S. and Miller, J.F.A.P. (1964). Nature, 204, 151.

McMaster, P. D. and Hudack, S. S. (1935). J. exp. Med., 61, 783.

Nachtigal, D., Greenberg, E. and Feldman, M. (1968). Immunology, 15, 343.

Nakano, M. and Braun, W. (1966). Science, 151, 338.

Naspitz, C. K. and Richter, M. (1968). Progr. Allergy, 12, 1.

Nordin, A. A. (1968). Proc. Soc. exp. Biol. Med., 129, 57.

Norman, A., Sasaki, M. S., Ottoman, R. E. and Fingerhut, A. G. (1965). Science, 147, 745.

Nossal, G.J.V. (1962). Ann. Rev. Microbiol., 16, 53.

Nossal, G.J.V. (1964). Ann. N.Y. Acad. Sci., 120, 171.

Nossal, G.J.V. (1965). Aust. Ann. Med., 14, 321.

Nossal, G.J.V., Ada, G. L. and Austin, C. M. (1964). Aust. J. exp. Biol. med. Sci., 42, 311.

Nossal, G,J.V., Austin, C. M., Pye, J. and Mitchell, J. (1966). Int. Arch. Allergy, 29, 368.

Nossal, G.J.V. and Mäkelä, O. (1962). J. exp. Med., 115, 209.

Nossal, G.J.V., Mitchell, J. and McDonald, W. (1963). Aust. J. exp. Biol. med. Sci., 41, 423.

Nossal, G.J.V., Shortman, K. D., Miller, J.F.A.P., Mitchell, G. F. and Haskill, J. S. (1967). Cold Spr. Harb. Symp. quant. Biol., 32, 369.

Nowell, P. C. (1960). Cancer Res., 20, 462.

Olsen, I. A. and Yoffey, J. M. (1967). In "The Lymphocyte in Immunology and Haemopoiesis". Yoffey, J. M. (ed.). Edward Arnold, London. p. 358.

Olson, G. B. and Wostmann, B. S. (1966). J. Immunol., 97, 267.

Oppenheim, J. J., Wolstencroft, R. A. and Gell, P.G.H. (1967). Immunology, 12, 89.

Osmond, D. G. and Everett, N. B. (1964). Blood, 23, 1.

Osoba, D. (1965a). J. exp. Med., 122, 633.

Osoba, D. (1965b). Science, 147, 298.

Osoba, D. (1968a). In "Regulation of the Antibody Response". Cinader, B. (ed.). Charles C. Thomas, Springfield. p. 232.

Osoba, D. (1968b). Proc. Soc. exp. Biol. Med., 127, 418.

Osoba, D. and Miller, J.F.A.P. (1963). Nature, 199, 653.

Osoba, D. and Miller, J.F.A.P. (1964). J. exp. Med., 119, 177.

Papermaster, B. W. (1967). Cold Spr. Harb. Symp. quant. Biol., 32, 447.

Parrott, D.M.V. (1962). Transplant. Bull., 29, 102.

Parrott, D.M.V. and East, J. (1964). In "The Thymus in Immunobiology. Structure, Function and Role in Disease". Good, R. A. and Gabrielsen, A. E. (eds.). Hoeber-Harper, New York. p. 523.

Parrott,D.M.V., Sousa, M.A.B. de and East. J. (1966). J. exp. Med., 123, 191.

Perey, D.Y.E., Cooper, M. D. and Good, R. A. (1968). Science, 161, 265.

Pernis, B., Chiappino, G., Kelus, A. S. and Gell, P.G.H. (1965). J. exp. Med., 122, 853.

Perry, S., Irvin, G. L. III and Whang, J. (1967). Blood, 29, 22.

Phillips, M. E. and Thorbecke, G. J. (1966). Int. Arch. Allergy, 29, 568.

Pinchuck, P., Fishman, M., Adler, F. L. and Maurer, P. H. (1968). Science, 160, 194.

Pinnas, J. L. and Fitch, F. W. (1966). Int. Arch. Allergy, 30, 217.

Playfair, J.H.L., Papermaster, B. W. and Cole, L. J. (1965). Science, 149, 998.

Pollard, M. (1966). Nat. Cancer Inst. Monogr., 20, 167.

Popp, R. A. (1960). Proc. Soc. exp. Biol. Med., 104, 722.

Porter, P. J., Spievack, A. R. and Kass, E. H. (1966). J. Lab. clin. Med., 68, 455.

Potworowski, E. F. and Nairn, R. C. (1967). Nature, 213, 1135.

Prescott, D. M. (1964). In "Methods in Cell Physiology. I". Prescott, D. M. (ed.). p. 365.

Pulvertaft, R.J.V. (1959). Proc. roy. Soc. Med., 52, 315.

Radovich, J., Hemingsen, H. and Talmage, D. W. (1968). J. Immunol., 100, 756.

Radovich, J. and Talmage, D. W. (1967). Science, 158, 512.

Rajewsky, K. and Rottlander, E. (1967). Cold Spr. Harb. Symp. quant. Biol., 32, 547.

Rebuck, J. W. (1947). Amer. J. clin. Path., 17, 614.

Reed, N. D. and Jutila, J. W. (1965). Science, 150, 356.

Reed, N. D. and Jutila, J. W. (1967). J. Immunol., 99, 238.

Rees, R.J.W. (1966). Nature, 211, 657.

Reese, A.J.M. and Israel, M. S. (1967). ibid, 215, 1085.

Reinhardt, W. O. (1964). Ann. N.Y. Acad. Sci., 113, 844.

Reinhardt, W. O. and Yoffey, J. M. (1956). Amer. J. Physiol., 187, 493.

Rieke, W. O. (1962). J. Cell Biol., 13, 205.

Rieke, W. O. (1964). Anat. Rec., 148, 327.

Rieke, W. O. (1966). Science, 152, 535.

Rieke, W. O. and Schwarz, M. R. (1967). In "The Lymphocyte in Immunology and Haemopoiesis." Yoffey, J. M. (ed.). Edward Arnold, London. p. 224.

Robinson, S. H. and Brecher, G. (1963). Science, 142, 392.

Robinson, S. H., Brecher, G., Lourie, I. S. and Haley, J. E. (1965). Blood, 26, 281.

Rogister, G. (1965). Transplantation, 3, 669.

Ross, M. H., Furth, J. and Bigelow, R. R. (1952). Blood, 7, 417.

Rowley, D. A., Fitch, F. W., Mosier, D. E., Salliday, S., Coppleson, L. W. and Brown, B. W. (1968). J. exp. Med., 127, 983.

Saffier, S. Cottier, H., Cronkite, E. P., Jansen, C. R., Rai, K. R. and Wagner, H. P. (1967). Blood, 30, 301.

Sainte-Marie, G. (1963). J. Immunol., 91, 840.

Sainte-Marie, G. (1965). ibid, 94, 172.

Sainte-Marie, G. (1966). J. cell. Physiol., 67, (Suppl. 1), 109.

Sainte-Marie, G. and Leblond, C. P. (1964). Blood, 23, 275.

Salomon, J. C. and Lecourt, J. C. (1966). Proc. Soc. exp. Biol. Med., 122, 640.

Salvin, S. B., Peterson, R.D.A. and Good, R. A. (1965). J. Lab. clin. Med., 65, 1004.

Santos, G. W. and Owen, A. H. Jr. (1966). Bull. Johns Hopk. Hosp., 118, 127.

Saunders, G. C. and King, D. W. (1966). Science, 151, 1390.

Schaller, R. T. Jr. and Stevenson, J. K. (1967). Proc. Soc. exp. Biol. Med., 124, 199.

Schierman, L. W. and McBride, R. A. (1967). Science, 156, 658.

Schlesinger, M. and Mark, R. (1964). ibid, 143, 965.

Schlesinger, M. and Hurvitz, D. (1968). J. exp. Med., 127, 1127.

Schooley, J. C. (1961). J. Immunol., 86, 331.

Schooley, J. C., Bryant, B. J. and Kelly, L. S. (1959). In "The Kinetics of Cellular Proliferation". Stohlman, F. Jr. (ed.). Grune and Stratton, New York. p. 208.

Schooley, J. C. and Kelly, L. S. (1964). In "The Thymus in Immunobiology. Structure, Function and Role in Disease". Good, R. A. and Gabrielsen, A. E. (eds.). Hoeber-Harper, New York. p. 236.

Schooley, J. C., Kelly, L. S., Dobson, E. L., Finney, C. R., Havens, V. W. and Cantor, L. N. (1965). J. Reticuloendothel. Soc., 2, 396.

Schooley, J. C. and Shrewsbury, M. M. (1967). In "The lymphocyte in Immunology and Haemopoiesis." Yoffey, J. M. (ed.). Edward Arnold, London. p. 366.

Schrek, R. and Batra, K. V. (1966). Lancet, 2, 444.

Schriever, H. G., Hsu, K. C. and Azar, H. A. (1967). Amer. J. Path., 50, 177.

Schwartz, R. S. (1965). Progr. Allergy, 9, 246.

Schwarz, M. R. (1966). Immunology, 10, 281.

Schwarz, M. R. (1968). Blood, 32, 225.

Schwarz, M. R. and Rieke, W. O. (1966). Anat. Rec., 155, 493.

Selye, H. (1936). Brit. J. exp. Path., 17, 234.

Sercarz, E. and Coons, A. H. (1962). In "Mechanisms of Immunological Tolerance". Hašek, M., Lengerová, A. and Vojtisková, M. (eds.). Academic Press, London. p. 73.

Shearer, G. M., Cudkowicz, G., Connell, M. St.J. and Priore, R. L. (1968). J. exp. Med., 128, 437.

Shelton, E. (1966). Amer. J. Anat., 119, 341.

Sherman, J. D. (1967). Arch. Path., 83, 251.

Sherman, J. D., Adner, M. M. and Dameshek, W. (1963). Blood, 22, 252.

Sherman, J. D., Adner, M. M. and Dameshek, W. (1964). Proc. Soc. exp. Biol. Med., 115, 866.

Shorter, R. G. and Bollman, J. L. (1960). Amer. J. Physiol., 198, 1014.

Shrewsbury, M. M. (1959). Proc. Soc. exp. Biol. Med., 101, 492.

Siegler, R. (1965). J. exp. Med., 122, 929.

Siminovitch, L., McCulloch, E. A. and Till, J. E. (1963). J. cell. comp. 62, 327.

Simonsen, M. (1962). Progr. Allergy, 6, 349.

Sinclair, N. R. St C. (1967). Immunology, 12, 559.

Sinclair, N. R. St C. and Elliott, E. V. (1968a). J. Immunol., 101, 251.

Sinclair, N. R. St C. and Elliott, E. V. (1968b). Immunology, 15, 325.

Sinclair, N. R. St C. and Millican, D. (1967). Clin. exp. Immunol., 2, 269.

Sjövall, H. (1936). Cited in Yoffey and Courtice (1956).

Small, M. and Trainin, N. (1967). Nature, 216, 377.

Smith, H. F. (1936). J. Coun. Sci. Indust. Res., 9, 211.

Smith, S. B., Isaković, K. and Waksman, B. H. (1966). Proc. Soc. exp. Biol. Med., 121, 1005.

Smithies, O. (1965). Science, 149, 151.

Somer, P. de, Denys, P. Jr. and Leyten, R. (1963). Life Sci., 2, 810.

Sosin, H., Hilgard, H. and Martinez, C. (1966). J. Immunol., 96, 189.

Spector, W. G., Walters, M. N-I. and Willoughby, D.A. (1965). J. Path. Bact., 90, 181.

Staples, P. J., Gery, I. and Waksman, B. H. (1966). J. exp. Med., 124, 127.

Stavitsky, A. B. (1958). Brit. J. exp. Path., 39, 46.

Šterzl, J. (1959). Nature, 183, 547.

Šterzl, J. (1961). Nature, 189, 1022.

Šterzl, J. and Jílek, M. (1967). ibid, 216, 1233.

Šterzl, J., Veselý, J., Jílek, M. and Mandel, L. (1965). In "Molecular and Cellular Basis of Antibody Formation". Šterzl, J. (ed.). Czech. Acad. Sci., Prague. p. 463.

Stoner, R. D. and Bond, V. P. (1963). J. Immunol., 91, 185.

Stoner, R. D. and Hale, W. M. (1955). ibid, 75, 203.

St Pierre, R. L. and Ackerman, G. A. (1965). Science, 147, 1307.

St Pierre, R. L. and Ackerman, G. A. (1966). Proc. Soc. exp. Biol. Med., 122, 1280.

Strober, S. (1968). Nature, 219, 649.

Stutman, O., Yunis, E. J. and Good, R. A. (1967). Lancet, 1, 1120.

Stutman, O., Yunis, E. J. and Good, R. A. (1968). Exp. hemat. No. 16. p. 18.

Stutman, O., Yunis, E. J., Teague, P. O. and Good, R. A. (1968). Transplantation, 6, 514.

Stutman, O. and Zingale, S. B. (1964). Proc. Soc. exp. Biol. Med., 117, 389.

Syeklocha, D., Siminovitch, L., Till, J. E. and McCulloch, E. A. (1966). J. Immunol., 96, 472.

Szenberg, A. and Cunningham, A. J. (1968). Nature, 217, 747.

Takeya, K. and Nomoto, K. (1967a). J. Immunol., 99, 831.

Takeya, K. and Nomoto, K. (1967b). Nature, 213, 1248.

Tallberg, T. and Kosunen, T. V. (1966). Ann. Med. exp. Biol. Fenn., 44, 24.

Tannenberg, W.J.K. (1967). Nature, 214, 293.

Taylor, R. B. (1963). Nature, 199, 873.

Taylor, R. B. (1968). Nature, 220, 611.

Thorbecke, G. J. and Cohen, M. W. (1964). In "The Thymus". Wistar Inst. Symp. Monogr. No. 2. Defendi, V. and Metcalf, D. (eds.). Wistar Inst. Press, Philadelphia. p. 33.

Thorbecke, G. J., Gordon, H. A., Wostman, B., Wagner, M. and Reyniers, J. A. (1957). J. infect. Dis., 101, 237.

Thorbecke, G. J. and Keuning, F. J. (1953). J. Immunol., 70, 129.

Till, J. E. and McCulloch, E. A. (1961). Radiat. Res., 14, 213.

Till, J. E., McCulloch, E. A., Phillips, R. A. and Siminovitch, L. (1967). Cold Spr. Harb. Symp. quant. Biol., 32, 461.

Trainin, N., Bejerano, A., Strahilevitch, M., Goldring, D. and Small, M. (1966). Isr. J. med. Sci., 2, 549.

Trainin, N., Burger, M. and Kaye, A. M. (1967). Biochem. Pharmacol., 16, 711.

Trainin, N., Law, L. W. and Levey, R. H. (1965). Proc. Soc. exp. Biol. Med., 118, 79.

Trainin, N. and Linker-Israeli, M. (1967). Cancer Res., 27, 309.

Trench, C.A.H., Watson, J. W., Walker, F. C., Gardner, P. S. and Green, C. A. (1966). Immunology, 10, 187.

Trowell, O. A. (1958). Int. Rev. Cytol., 7, 235.

Tyan, M. L. and Cole, L. J. (1964). Transplantation, 2, 241.

Tyan, M. L., Cole, L. J. and Nowell, P. C. (1966). ibid, 4, 79.

Uhr, J. W. and Möller, G. (1968). Advanc. Immunol., 8, 81.

Urso, P. and Makinodan, T. (1963). J. Immunol., 90, 897.

Uyeki, E. M. (1967). Proc. Soc. exp. Biol. Med., 120, 115.

Vanderputte, M. (1967). Path. Europ., 2, 55.

Virolainen, M. (1968). J. exp. Med., 127, 943.

Volkman, A. (1966). ibid, 124, 241.

Volkman, A. and Gowans, J. L. (1965a). Brit. J. exp. Path., 46, 50.

Volkman, A. and Gowans, J. L. (1965b). ibid, 46, 62.

Vos, O., Vries, M. J. de, Collenteur, J. C. and Bekkum, D. W. van (1959). J. nat, Cancer Inst., 23, 53.

Vries, M. J. de, Putten, L. M. van, Balner, H. and Bekkum, D. W. van (1964). Rev. Franc. Etudes. clin. Biol., 9, 381.

Wagner, H. P., Cunningham, L., Cottier, H., Jansen, C. R., Rai, K. R. and Cronkite, E. P. (1967). Exp. Cell Res., 46, 441.

Waksman, B. H. (1964). Ann. N.Y. Acad. Sci., 116, 1045.

Waksman, B. H., Arnason, B. G. and Jankovič, B. D. (1962). J. exp. Med., 116, 187.

Warner, N. L. (1964). Aust. J. exp. Biol. med. Sci., 42, 401.

Warner, N. L. (1965). ibid, 43, 439.

Warner, N. L. (1967). Folia Biol. (Praha), 13, 1.

Warner, N. L. and Szenberg, A. (1962). Nature, 196, 784.

Weber, W. T. (1966a). J. cell. Physiol., 67, 285.

Weber, W. T. (1966b). ibid, 68, 117.

Weber, W. T. (1967). Exp. Cell Res., 46, 464.

Weiss, L. and Miller, J.F.A.P. (1966). Fed. Proc., 25, 613.

Weissman, I. L. (1967). J. exp. Med., 126, 291.

Whitelaw, D. M. (1965). Blood, 25, 749.

Wigzell, H. (1967). Cold Spr. Harb. Symp. quant. Biol., 32, 507.

Wigzell, H., Möller, G. and Andersson, B. (1966). Acta path. microbiol. scand., 66, 530.

Wigzell, H. and Stjernswärd, J. (1966). J. nat. Cancer Inst., 37, 513.

Wilson, D. B. and Billingham, R. E. (1967). Advanc. Immunol., 7, 189.

Wilson, R., Bealmear, M. and Sobonya, R. (1965). Proc. Soc. exp. Biol. Med., 118, 97.

Wilson, R., Sjodin, K. and Bealmear, M. (1964). Proc. Soc.exp. Biol. Med., 117, 237.

Winkelstein, A. and Craddock, C. G. (1967). Blood, 29, 594.

Wissler, R. W., Fitch, F. W., LaVia, M. F. and Gunderson, C. H. (1957). J. cell. comp. Physiol., 50 (Suppl. 1), 265.

Wolf, B. (1968). Immunology, 14, 235.

Wong, F. M., Taub., R. N., Sherman, J. D. and Dameshek, W. (1966). Blood, 28, 40.

Woodruff, J. and Gesner, B. M. (1968). Science, 161, 176.

Wortis, H. H., Taylor, R. B. and Dresser, D. W. (1966). Immunology, 11, 603.

Yoffey, J. M. and Drinker, C. K. (1939). Anat. Rec., 73, 417.

Yoffey, J. M. and Courtice, F. C. (1956). "Lymphatics, Lymph and Lymphoid Tissue". Edward Arnold, London.

Young, I. and Friedman, H. (1967). In "Germinal Centres in Immune Response". Cottier, H., Odartchenko, N., Schindler, R. and Congdon, C. C. (eds.). Springer-Verlaag, New York. p. 102.

Yunis, E. J., Hilgard, H. R., Martinez, C. and Good, R. A. (1965). J. exp. Med., 121, 607.

Yunis, E. J., Hilgard, H., Sjodin, K., Martinez, C. and Good, R. A. (1964). Nature, 201, 784.

Yunis, E. J., Martinez, C. and Good, R. A. (1964). ibid, 204, 664.

Yunis, E. J., Martinez, C. and Good, R. A. (1967). Proc. Soc. exp. Biol. Med., 124, 418.

Yunis, E. J., Martinez, C., Smith, J. and Good, R. A. (1964). Nature, 204, 850.

Zaalberg, O. B. (1964). ibid, 202, 1231.

Zaalberg, O. B., Meul, V. A. van der and Twisk, J. M. van (1966). ibid, 210, 544.

Zinzar, S. N. and Svet-Moldavsky, G. J. (1967). ibid, 214, 295.